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SCIENTIFIC RATIONALE FOR THE SELECTION OF TOXICITY TESTING METHODS: HUMAN HEALTH ASSESSMENT

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SCIENTIFIC RATIONALE FOR THE SELECTION OF TOXICITY TESTING METHODS: HUMAN HEALTH ASSESSMENT

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Health and Environmental Studies Program
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FOREWORD

This report was prepared by the Health and Environmental Studies Program (HESP), Information Center Complex, Information Division, Oak Ridge National Laboratory, under an interagency agreement between the U.S. Department of Energy and the U.S. Environmental Protection Agency (EPA). Under this agreement HESP is providing a variety of technical information support services to the EPA Office of Testing and Evaluation, including: performing technical review and follow-up on chemical studies, arranging workshops, writing reviews on health effects of specific chemicals, and conducting structure-activity oriented reviews of chemicals associated with mutagenic or teratogenic effects.

This document is part one of a two-part literature analysis of parameters associated with the various toxicity testing methods. The information contained herein has been compiled for the purpose of assisting and supporting EPA in its efforts to develop guidelines for more efficient and more economical toxicity testing procedures.

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ABSTRACT

This document is the first of a two-part literature analysis of parameters associated with the various toxicity testing methods (test animal selection, pathology requirements, etc.). Acute, subchronic, chronic, and carcinogenic testing methods are covered; a discussion of some basic experimental considerations is also included. This report was prepared for the purpose of assisting and supporting the U.S. Environmental Protection Agency in its efforts to develop guidelines for more efficient and economical testing procedures.

1. SUMMARY

1.1 BASIC EXPERIMENTAL CONSIDERATIONS

To assist in the development of test standards, this section addresses certain aspects of test materials, husbandry, diet, pathological examination, and pharmacokinetics.

The chemical and physical properties of the test chemical should be known prior to conducting a toxicity test of a chemical. The chemical and physical properties will influence laboratory storage practices and will permit the researcher to determine the stability of the chemical under the conditions of the experiment. In addition, the chemical purity of the test material and possible interactions with the vehicle may be investigated.

Husbandry conditions associated with toxicity testing can affect the test results and should be controlled or standardized. Included among the husbandry factors are animal selection, transportation, quarantine, disease control, cage design, test facility design, regulation of macroclimate and microclimate variables, and experienced technical personnel.

Nutrients required by laboratory animals are supplied through various types of diets. Variations in the concentration of essential nutrients and the presence of contaminants in the diet may affect the response of test animals to toxic substances. Periodic testing of diets for nutrient concentrations and contaminants has been proposed.

Pathology is a very essential part of the evaluation of a chemical's toxicity, providing information on the nature of the lesions and indicating dose-effect relationships. A successful pathology evaluation should include the following: (1) the pathologist should perform or supervise all steps in the protocol; (2) the gross necropsy should be extremely thorough and include every animal in the study; (3) the tissues should be properly preserved in an accepted fixative immediately after the necropsy; (4) the staining, embedding, and sectioning of the tissues must be well planned and coordinated, especially if special stains are utilized; and (5) the microscopic examination should be as thorough as practical limitations will allow.

The pharmacokinetics of a chemical, which is influenced by four major rate processes — absorption, distribution, metabolism, and elimination can provide valuable information to the researcher. The results of pharmacokinetic studies can help decide the future testing protocol for a chemical as well as explain why a chemical can be teratogenic when administered during one part of the gestation period but not teratogenic when administered in another part of the gestation period.

1.2 ACUTE TOXICITY TESTING

Acute toxicity studies are very useful because they provide baseline information such as the LD_{50} (the most frequently determined index of toxicity), the relative sensitivity of the various species, and the comparative toxicity of various chemicals. They also identify the nature of the toxicity or the mode of the toxic action and provide guidance on doses to be used in subsequent experiments. Administration may be a single oral or single parenteral injection or may be given in food or drinking water over a 24-h period. When the laboratory rat is used as the test animal, both sexes should be tested because of the possibility of sex-related differences in LD_{50} values.

The time following dosing, during which deaths are recorded for the purpose of determining an LD_{50}, is the observation period. This period must be of sufficient duration for manifestation of delayed effects but not so long that there is doubt whether the animal died as a direct result of the test substance. The 7- and 14-day observation periods, particularly the 14-day period, seem to be the most common lengths for observation periods among researchers in the United States. Researchers in other countries use observation periods of various lengths. Close clinical observation of the animal is necessary to assist in the assessment of the toxic effects.

1.3 SUBCHRONIC TOXICITY TESTING

Subchronic tests are designed to provide two types of toxicity data. As a precursor to a chronic study, subchronic tests should identify target organs and define appropriate dose ranges for long-term exposures. When used as the primary evaluation of the effects of chronic exposure, subchronic tests can provide detailed information on toxicity and the potential hazard of the chemical to man. The parameters to be employed to assess toxicity vary with the purpose of the test; they are usually more complex when the subchronic test is to be the primary evaluation of long-term effects.

The number of species employed in a subchronic test is an area under discussion in the protocol design. Most information suggests that in a subchronic test both a rodent and nonrodent species should be used. The overlap provided by the use of two mammals increases the efficiency of hazard assessment for human exposure. In general the rat and dog appear to be the species most often employed in these hazard assessments. However, whenever possible the choice of species should be governed by information on pharmacokinetics and metabolism of the compound. Under such circumstances other species might also be considered.

The duration of exposure for a subchronic test is another area under discussion. The literature suggests that a 90-day exposure can provide essentially the same toxicity data as would be provided by a long-term test, excluding carcinogenicity and teratogenicity. This information could be the basis for regulatory decisions. However, there is additional information in the literature suggesting that a 30-day duration might be acceptable. The comparisons available indicate that in many cases the effects can be predicted qualitatively but not quantitatively. This question needs more data for analysis. Comparison studies or access to unpublished information in industry files is needed to fully resolve the debate over the minimum duration necessary for a subchronic test.

The routes of exposure for a subchronic test should ideally represent the expected routes of human exposure. Most often this is by ingestion or inhalation, which are the principal exposure routes utilized in test designs. Oral exposure can be achieved relatively easily, but inhalation exposure is more complicated, requiring special test facilities. Extrapolation of data obtained from one route to evaluate the hazard with exposure to another route could provide a solution to the problems of inhalation tests. However, there is little information on the criteria necessary for or the hazards involved in such an extrapolation. Much more research is needed to assess the predictability and feasibility of route-to-route extrapolations.

In subchronic testing much use is made of biochemical tests and pathology as parameters to evaluate toxicity. Both biochemical tests and pathology can provide data to assess toxicity.

Pathological evaluations should include a gross examination of all organs of all test animals at all dose levels. Organ weights should also be recorded for the major tissues. Microscopic examination, because of its higher costs, may be limited to the evaluation of selected tissues at high and control dose levels. Additionally, any lesion-bearing organs, detected in the gross examination, should be examined microscopically. Further microscopic examination at other dose levels should be based on the pathology results from the high dose level examination.

Biochemical tests should be employed to provide information on subtle changes and to fully assess the extent of toxic effects. A well-designed subchronic study should include a basic hematology examination (evaluating cellular damage and hemorrhaging), a set of enzyme and organ function tests (primarily evaluating liver and kidney damage), and a urinalysis examination (primarily for kidney damage). Changes indicated by these tests can be investigated further by more complex biochemical tests.

1.4 CHRONIC TOXICITY AND CARCINOGENICITY TESTING

Many factors influence the results of chronic toxicity and carcinogenicity tests in animals, including route of administration, dosage and frequency of exposure, species, strain, sex, age of the animal when the test is initiated, dietary constituents, immunologic status of the animal, duration of the experiment, and other factors.

The principal objective for conducting chronic toxicity tests is to determine the toxic potential of a chemical when it is administered for the greater part of the life span of the animal. In testing of chemicals for their carcinogenic potential the major objective may be simply to determine whether or not the chemical is capable of inducing tumors in a particular strain or species of animals.

Because most chronic toxicity and carcinogenicity tests are conducted over the major portion of the life span of the animal, short-lived rodents are the choice of most investigators. However, the dog is occasionally used, particularly in tests for chronic toxicity.

Test duration is an issue under discussion. Attempts to standardize this parameter have been unsuccessful. Generally, when mice and rats are used for chronic toxicity and carcinogenicity tests, 24 to 27 months and approximately 24 months are recommended, respectively. Typically, dogs are treated for 1 to 2 years in chronic toxicity studies.

New strains of animals that are especially susceptible to tumor induction and genetic factors that influence the spontaneous tumor incidences in laboratory animals have been exploited in the development of certain short-term assays. These and other in vivo short-term bioassays could be considered as intermediate between in vitro short-term studies and lifetime studies and may become valuable tools in screening potential carcinogens.

The number of test animals used is a compromise between requirements for good statistical precision and reasonable costs and work loads. Most authorities now recommend 50 rodents or four to eight nonrodents per test group. Concurrent control groups comprising animals of the same species, age, sex, weight, and number are also required.

In tests for chronic toxicity at least three dose levels should be used, and both treatment and observation of animals should be conducted 7 days per week. In tests for carcinogenicity in which induction of tumors is the primary end point, two or three doses of the test chemical may be employed. If information on dose-response relationships is required, then several doses are necessary to provide data for appropriate statistical analysis.

Interim sacrifices may be performed to provide information on the progression of toxic events. Macroscopic examination of animals and microscopic examination of tissues are necessary in evaluating the hazard of chemical exposure. As a minimum, all major organs and tissues of high dose and control animals are recommended for histological examination. This study should also include any other tissues exhibiting gross lesions.

2. BASIC EXPERIMENTAL CONSIDERATIONS

2.1 INTRODUCTION

This section will briefly discuss some basic experimental parameters, such as the purity and chemical stability of the test chemical; the importance of selecting the appropriate vehicle; the diet, husbandry, and pathological examination of the test animal; and the importance of pharmacokinetics in the toxicological evaluation of chemicals. The discussions will illustrate why careful attention to each of these is essential to accurate and reliable experimental results.

2.2 TEST MATERIALS

2.2.1 Introduction

The chemical and physical properties of the test material must be known prior to the initiation of a toxicity experiment. This is essential because the toxicologist must know that the results obtained from his experiment are caused by the compound under investigation and not by a degradation or contamination product. The scientific committee of the Food Safety Council in agreement with this states that the "lack or disregard of information on the chemical nature of the material to be tested not only limits the usefulness of toxicological data for regulatory purposes but can, in some instances, provide an erroneous impression of its toxicity" (Food Safety Council, 1978). A document published by the authority of the Ministry of Health and Welfare Canada (1975) further states that, before a long-term study is initiated, batch-to-batch variations of the compound, chemical synthetic processes, packaging and handling procedures, storage requirements, and interactions with other chemicals must be considered. The following subsections will briefly describe some aspects of the pretesting examination of the test material.

2.2.2 Chemical Stability

As soon as a substance arrives in the toxicology laboratory, the stability of the chemical at various pH values and its photochemical properties must be determined (World Health Organization, 1978). These parameters may determine the manner in which the chemical should be stored prior to use. The stability of the test material under the same conditions in which it will be administered must also be determined (Sontag, Page, and Saffiotti, 1976). If the chemical nature of the test substance changes during an experiment as a result of the experimental conditions, the results will not be reliable. In a worst-case situation, the chemical stability of the test material could be altered without the researcher's knowledge, thus permitting incorrect conclusions concerning the action of the test substance. Therefore, prior to the start of the toxicity test the stability of the test chemical in the feed and/or vehicle, at the temperature and/or pH to be used in the test, must be investigated to ensure that the chemical stability is not altered by the experimental conditions. The stability of the test material under its conditions of storage and/or administration will influence the frequency at which fresh treatment mixtures are prepared (National Academy of Sciences, 1975; Sontag, Page, and Saffiotti, 1976).

When the test material is a chemical used in food processing, the possibility exists that under conditions of use the chemical may be transformed or degraded in foods, with the resulting products being either more or less toxic than the original chemical (Food Safety Council, 1978). The knowledge of potential chemical transformation would be essential for the safety assessments of chemicals in foods.

2.2.3 Chemical Impurities

The chemical purity of the test material should be established prior to toxicity testing (Sontag, Page, and Saffiotti, 1976). This is important because of the possiblity that trace contaminants could be responsible for, or at least modify, the observed biological effects attributed to the test material under investigation. As pointed out by the World Health Organization (1978), if the contaminants are unknown or their biological activity unsuspected, toxicity tests may lead to erroneous conclusions concerning the test material.

A related issue that confronts the toxicologist and is one of the earliest and most difficult decisions to be made is the selection of the desired purity of the sample to be studied (technical grade, highly purified, etc.) (World Health Organization, 1978). If the test material is a commercial product that is known to contain impurities, toxicity testing of a highly purified sample could result in erroneous conclusions. If the impurities are standardized, then testing of the commercial product would be the answer; however, for those commercial products, chemicals used in manufacturing, or chemicals incidentally released into the environment for which the impurities are not standardized, the toxicologist must make a decision concerning test sample purity.

2.2.4 Vehicles

The choice of the appropriate vehicle is important because the toxic activity of the test chemical can be altered, especially if the test chemical reacts chemically with the vehicle (also mentioned in Sect. 2.2.2). However, the toxic activity of the test chemical can be altered without a change in chemical structure. One example is that of cottonseed oil, which, unless refined by superheated-steam processing, may contain cyclopropenoid fatty acids, which can enhance the activity of some carcinogens (Lee et al., 1968, as cited by National Academy of Sciences, 1975). Other examples of vehicle interference in tests for carcinogenicity are described by Pott, Brockhaus, and Huth (1973), who observed that when a subcutaneous dose of benzo(*a*)pyrene was kept constant, increasing the dose of the vehicle, tricoprylin, increased the rate of tumor production, and by Mori (1965), who reported an increase in organs affected for tumor induction when 4-nitroquinoline-1-oxide was administered in olive oil and lecithin instead of in a solvent mixture containing cholesterol.

2.2.5 Homogeneity in Vehicle

If a text mixture is prepared in sufficient quantity for the dosing of several animals that are to receive the same concentration of the test material, the homogeneity in the vehicle is essential. Sontag, Page, and Saffiotti (1976) state that in a long-term study where the test material is incorporated into the feed, the homogeneity and concentration in the diet mix should be determined before the start of the study and periodic analysis of random samples from freshly mixed batches should be performed to ensure that proper mixing and formulation procedures are being used.

2.2.6 Conclusions

Prior to the initiation of an experiment designed to evaluate the toxicity of a chemical, consideration should be given to the characteristics of the test chemical and the vehicle. Specifically, the stability and the purity of the test chemical should be known, and the possibility of the vehicle altering the test results must be considered.

2.3 HUSBANDRY

2.3.1 Introduction

In all types of toxicity tests, the results can be influenced by the husbandry conditions under which the testing is performed. Husbandry factors that should be managed include selection, transportation, and quarantine of the test animals; control of diseases; proper design of the cages and testing facility; regulation of the temperature, humidity, light, ventilation, noise, and handling; and certification of personnel. Unless these factors are standardized or controlled, the data obtained cannot be used with any certainty to evaluate the potential harmful effects of the chemical to man. The following subsections will briefly discuss these factors.

2.3.2 Animal Selection

In addition to the consideration of species and human metabolic similarity discussed elsewhere in the text, several important factors should be considered or controlled when selecting animals for use in toxicity studies. First, all animals should have standardized life cycle variables, such as age, sexual maturity, and mating status, as these factors can affect toxicity (Hurni, 1970). Next, the genetic composition should be uniform, because this ensures consistency of response and reproducibility of results (Food Safety Council, 1978; Zbinden, 1963). The current breeding methods for maintaining a constant genotype include inbred, outbred, and hybrid strains. Inbred animals are crosses of brother and sister or parent and offspring that have been maintained for at least 20 generations (National Academy of Sciences, 1969). This results in a specific homozygous genotype where all animals in the colony are as similar as identical twins (Hurni, 1970). Outbred or random bred strains maintain an unaltered, heterozygous pool of genetic material on a population level (Hurni, 1970) but are rarely used in toxicity studies (National Academy of Sciences, 1969). Because both of these options have problems, hybrids from crosses of inbred strains are often utilized. These animals are genetically uniform but are not homozygous (Food Safety Council, 1978; Hurni, 1970). Festing (1979) reviews, in more detail, the pros and cons of inbred, outbred, and hybrid animals in toxicity testing.

The need for reduction of animal health as a factor stimulated the development and use of specific pathogen-free (SPF) strains. These are specially bred animals (best obtained by aseptic hysterectomy and artificial rearing of germ-free derived animals) that have accepted levels of health and are known to be free of certain standard diseases and parasites (Hurni, 1970; Meister, Hobik, and Metzger, 1967). However, these animals require special isolated colonies with "clean zone" barriers and can be difficult to maintain (Hurni, 1970). Therefore, as with other factors, the nature of the experiment and the risks involved will decide the animal type and quality to be used (Page, 1977).

2.3.3 Transportation, Quarantine, and Disease Control

After selection of the appropriate test animals, transportation to the testing facility or animal room is necessary. This can affect the response of the animals by subjecting them to unusual stresses, and they should be given time to recover (National Academy of Sciences, 1969). Heinecke (1967) has shown that even transport between buildings (e.g., from an in-house breeding colony to the testing facility) has affected the blood status of mice for 3 to 4 days afterwards. More extensive transportation can disrupt the normal state for up to 6 weeks (Hurni, 1970). Therefore, it is necessary to allow the animals to readjust and acclimate before initiating testing.

While the animals are acclimating, they can be kept isolated from the rest of the facility for quarantine purposes (Page, 1977). The quarantine is necessary to prevent the introduction of diseases into the existing facility population. The duration of quarantine varies with the species, its source, and the testing purpose (U.S. Department of Health, Education, and Welfare, 1974), but should be at least 2 weeks (National Academy of Sciences, 1969). As part of the quarantine process, it is a good practice to include: (1) a physical examination upon arrival; (2) veterinary care to check for parasites and disease and to allow for immunization; (3) general grooming; and (4) a pathological examination of a small number of the test animals (Sontag, Page, and Saffiotti, 1976; U.S. Department of Health, Education, and Welfare, 1974). If any animals in quarantine are found to have a communicable disease, the whole group should be destroyed (Sontag, Page, and Saffiotti, 1976).

The quarantine of animals upon arrival is just part of a general need to control disease in the test facility. The presence of disease in the test facility can invalidate the test results, especially if the disease produces subtle, nonspecific, or long-term effects (van der Waaij and van Bekkum, 1967). To control disease, several steps are necessary: (1) acquire and include (after quarantine) only healthy animals; (2) prevent introduction of pathogens into test facility; (3) maintain sanitary conditions; and (4) utilize a well-planned disease control program (Flynn, 1967; National Academy of Sciences, 1969). The prevention of pathogen introduction should include monitoring and/or control of water supply, air supply, feed and bedding quality, and personnel contact (National Academy of Sciences, 1969). The use of filters and filter caps is particularly helpful to control airborne diseases (Clough, 1976). Sanitary control should include facility and personnel cleanliness, proper waste disposal practices, and vermin control (U.S. Department of Health, Education, and Welfare, 1974). The disease control program can be the most vital part of the process and should (1) be specific for that situation; (2) be aimed at the most common or expected diseases; and (3) include frequent observation by appropriately trained personnel (Flynn, 1967; U.S. Department of Health, Education, and Welfare, 1974). A detailed example of a disease control program is discussed by Flynn (1967). Fox (1977) reviewed some of the most common diseases, for each organ system, that occur in testing facilities.

2.3.4 Design of Testing Facility

The design, scope, and size of a toxicity testing facility is the product of many compromises, depending on the nature of the research, the number of test animals to be housed, and the types of species used (U.S. Department of Health, Education, and Welfare, 1974). The facility should be designed to provide maximum comfort and safety for the test animals and personnel, to minimize disease factors, and to minimize costs (National Academy of Sciences, 1969). It is essential that a facility design include a building, wing, floor, or room for the animals that is separate from human work areas; specialized laboratories for maintenance and assessment of the animals; a supply-receiving area; a quarantine area; and an incinerator (U.S. Department of Health, Education, and Welfare, 1974). Specific construction details are discussed in several reviews (Grange, 1976; National Academy of Sciences, 1969; U.S. Department of Health, Education, and Welfare, 1974).

In general the rooms should be arranged to provide barriers between individual rooms and the outside environment (National Academy of Sciences, 1969; Sontag, Page, and Saffiotti, 1976). There are three common types of control systems used in test facilities: open, closed, and isolated (Shaw, 1976). The open system does not have hermetically sealed rooms, and the entering of materials and personnel is not controlled. The closed system is hermetically sealed with sterilization controls over entering materials and personnel. An isolated system is similar to a closed one, but does not allow personnel to enter. One special type of control system is the "clean-dirty" system, which utilizes a unidirectional corridor flow of materials and personnel (Sontag, Page, and Saffiotti, 1976). Each room has a separate entrance and exit door, allowing personnel and materials to be decontaminated before entering and to leave only through the exit door (Page, 1977). This system is especially useful for minimizing disease contamination (Sontag, Page, and Saffiotti, 1976). If possible, there should also be individual rooms for each species and each test treatment. A separate room for controls, to evaluate the influence of various factors of the test facility, has also been suggested (Food Safety Council, 1978). The overall goal of the facility design is to reduce or standardize the environmental factors affecting test results in the most efficient manner.

2.3.5 Caging

The cage is the single most important element of the physical environment for laboratory animals since it represents the immediate barrier between the microenvironment and macroenvironment of the testing facility (Clough, 1976; U.S. Department of Health, Education, and Welfare, 1974). The criteria to evaluate a good cage design include: (1) it should meet the investigator's research requirements; (2) it should be designed with the animal's physical comfort as a prime consideration and; (3) it should be compatible with proper maintenance requirements, especially health and sanitary procedures (U.S. Department of Health, Education, and Welfare, 1974). Some important features that a good cage design incorporates are: (1) it should be stackable or fit on racks; (2) it should have smooth solid walls and be accessible for cleaning; (3) it should be constructed of stainless steel or plastic; (4) it should have a perforated bottom (depending on species or experiment requirements); and (5) it should have accessible feed and water sources (usually built into a removable lid) (National Academy of Sciences, 1969). Stainless steel and especially hard plastics are used for cage designs, because they are lighter and resistant to corrosion (Clough, 1976). A solid cage design offers more control over the microclimate (usually increasing humidity, NH_3, CO_2, and temperature levels) and affords more protection from microbial agents than wire mesh designs (Fox, 1977; Hurni, 1970). However, animals in solid cages require transfer to a clean cage at least every week, while animals in wire mesh cages can last 2 weeks (Sontag, Page, and Saffiotti, 1976). Also Winter and Flataker (1962) found that rats in solid cages developed more resistance to chemicals than those in wire mesh. Therefore, the choice of exact cage design is dependent on the goals and circumstances of the specific study.

The number of animals per cage and the amount of space for each are also determined by practical and experimental considerations. Hatch et al. (1965) found that a single mouse per cage was more susceptible to certain cancers than groups of 1 to 5 mice. Hurni (1970) discussed several studies in which isolated animals showed evidence of an increase in toxicity. However, grouping of animals in cages can indirectly affect toxicity, for example, by increasing food competition or fecal/urinary contamination (Food Safety Council, 1978). Table 2.1 gives some recommended space standards for routine housing of laboratory animals (U.S. Department of Health, Education, and Welfare, 1974). Table 2.2 lists the minimum number of square inches necessary per rodent and the maximum number of animals per cage (National Academy of Sciences, 1969). These guidelines address practical considerations, but the nature of the experiment often dictates the actual numbers of animals per cage.

2.3.6 Bedding

Bedding material is frequently used in housing test animals, especially rodents, and has been shown to affect toxicity. In general, bedding serves several purposes: (1) it provides thermal insulation;

Table 2.1. Space recommendations for laboratory animals

Species	Weight	Type of housing	Floor area per animal (squared)	Height[a]
Mouse	Up to 10 g	Cage	39 cm (6 in.)	12.7 cm (5 in.)
	10 to 15 g	Cage	52 cm (8 in.)	12.7 cm (5 in.)
	16 to 25 g	Cage	77 cm (12 in.)	12.7 cm (5 in.)
	Over 25 g	Cage	97 cm (15 in.)	12.7 cm (5 in.)
Rat	Up to 100 g	Cage	110 cm (17 in.)	17.8 cm (7 in.)
	100 to 200 g	Cage	148 cm (23 in.)	17.8 cm (7 in.)
	201 to 300 g	Cage	187 cm (29 in.)	17.8 cm (7 in.)
	Over 300 g	Cage	258 cm (40 in.)	17.8 cm (7 in.)
Hamster	Up to 60 g	Cage	64.5 cm (10.0 in.)	15.2 cm (6 in.)
	60 to 80 g	Cage	83.9 cm (13.0 in.)	15.2 cm (6 in.)
	81 to 100 g	Cage	103.2 cm (16.0 in.)	15.2 cm (6 in.)
	Over 100 g	Cage	122.6 cm (19.0 in.)	15.2 cm (6 in.)
Guinea pig	Up to 250 g	Cage	277 cm (43 in.)	17.8 cm (7 in.)
	250 to 350 g	Cage	374 cm (58 in.)	17.8 cm (7 in.)
	Over 350 g	Cage	652 cm (101 in.)	17.8 cm (7 in.)
Rabbit	Up to 2 kg	Cage	0.14 m (1.5 ft)	35.6 cm (14 in.)
	2 to 4 kg	Cage	0.28 m (3.0 ft)	35.6 cm (14 in.)
	Over 4 kg	Cage	0.37 m (4.0 ft)	35.6 cm (14 in.)
Cat	Up to 4 kg	Cage	0.28 m (3.0 ft)	61.0 cm (24 in.)
	Over 4 kg	Cage	0.37 m (4.0 ft)	61.0 cm (24 in.)
Dog[b]	Up to 15 kg	Pen or run	0.74 m (8.0 ft)	
	15 to 30 kg	Pen or run	1.12 m (12.0 ft)	
	Over 30 kg	Pen or run	2.23 m (24.0 ft)	
	Up to 15 kg	Cage	0.74 m (8.0 ft)	81.3 cm (32 in.)
	15 to 30 kg	Cage	1.12 m (12.0 ft)	91.4 cm (36 in.)
	Over 30 kg	Cage	[b]	[b]
Primates[c,d]				
Group 1	Up to 1 kg	Cage	0.15 m (1.6 ft)	50.8 cm (20 in.)
Group 2	Up to 3 kg	Cage	0.28 m (3.0 ft)	76.2 cm (30 in.)
Group 3	Up to 15 kg	Cage	0.40 m (4.3 ft)	76.2 cm (30 in.)
Group 4	Over 15 kg	Cage	0.74 m (8.0 ft)	91.4 cm (36 in.)
Group 5	Over 25 kg	Cage	2.33 m (25.0 ft)	213.4 cm (84 in.)

[a] Height means from the resting floor to the cage top.

[b] These recommendations may require modifications according to the body conformations of particular breeds. As a further general guide, the height of a dog cage should be equal to the height of the dog over the shoulders (at the withers), plus at least 6 inches, and the width and depth of the cage should be equal to the length of the dog from the tip of the nose to the base of the tail, plus at least 6 inches.

[c] The primates are grouped according to approximate size with examples of species that may be included in each group: Group 1 — marmosets, tupaias, and infants of various species; Group 2 — cebus and similar species; Group 3 — macaques and large African species; Group 4 — baboons, monkeys larger than 15 kg, and adult members of brachiating species such as gibbons, spider monkeys and woolly monkeys; Group 5 — great apes.

[d] Where primates are housed in groups in pens, only compatible animals should be kept. Minimum height of pens should be 6 feet. Resting perches, nesting boxes, and escape barriers necessary for the well-being of the particular animals should also be provided.

Source: Adapted from U.S. Department of Health, Education, and Welfare, 1974.

Table 2.2. Cage space requirements for rodents

	Minimum square inches per animal[a]	Maximum number of animals per cage
Mice		
Weaning to 5 weeks	6	40
5 to 8 weeks	8	30
8 to 12 weeks	12	20
Over 12 weeks	15	20
Hamsters		
Weaning to 5 weeks	10	20
5 to 10 weeks	12.5	16
Over 10 weeks	15	13
Rats		
Up to 50 g	15	50
50 to 100 g	17	50
100 to 150 g	19	40
150 to 200 g	23	40
200 to 300 g	29	30
Over 300 g	40	25
Guinea pigs		
Weaning to 350 g	60	15
350 g and over	90	10
Breeders	180	5

[a] In many circumstances, more space per animal may be needed.

Source: Adapted from National Academy of Sciences, 1969.

(2) it absorbs fecal and urinary wastes and water spillage; (3) it is used to build nests; and (4) it reduces stress by providing a protective, isolating cover (National Academy of Sciences, 1969). The material selected should therefore be absorbant, nonedible, and innocuous but not dusty or highly resinous (Clough, 1976; Hurni, 1970; National Academy of Sciences, 1969). Some of the bedding materials rated as acceptable by the National Academy of Sciences (1969) include: coarse pine sawdust; pine, cedar, basswood, or poplar shavings; crushed corn cobs; and hardwood chips. However, other researchers have found problems with some of these materials. Porter (1967) found that sawdust often contains many possible contaminants, and he preferred sterilized pine shavings. In contrast, Clough (1976) and Fox (1977) warned that pine, cedar, and other softwoods, if fresh, can induce biological variations in the

microsomal metabolizing enzymes and alter chemical toxicity. Hurni (1970) warned that hardwood shavings, which are high in tannic acid content, can lead to constipation in rodents. However, Sontag, Page, and Saffiotti (1976) preferred hardwood shavings for long-term carcinogenicity studies. In any case, the material must be sterilized, preferably by autoclaving, and be changed frequently.

2.3.7 Temperature and Humidity

Temperature and humidity are two environmental factors that should be controlled by the experimenter, since they can influence the test results. The effect of changes in these factors will be influenced by the magnitude, frequency, and duration of the changes, by whether or not the test animals have behavioral modifications to adapt to these, and by the current physical and health status of the animals (Weihe, 1976*a*). The easiest solution is to control temperature and humidity by air conditioning each animal room (U.S. Department of Health, Education, and Welfare, 1974).

Weihe (1964, as cited by Heinecke, 1967), by varying the environmental temperature, was able to affect the LD_{50}s of chemicals. Heinecke (1967) found that temperature changes also affected blood values. Hurni (1970) reviewed several studies in which cold temperatures or increases in ambient temperature affected both the resistance of test animals and the toxic effects of chemicals. Changes in ambient temperature can affect the metabolism of chemicals, and the effects produced can mimic chemical effects, making diagnosis difficult (World Health Organization, 1978). Humidity levels can also affect toxicity and were found to be a principal factor of ringtail in young rats (Clough, 1976; Flynn, 1967). Thus it is very important to control these factors.

The exact limits of temperature and humidity beyond which significant metabolic adaptation will occur are not known, but if the changes are moderate (±5°K or 20% to 30% humidity) the animals should be able to adapt behaviorally without damage (Weihe, 1976*a*). The National Academy of Sciences (1969) recommends a fluctuation of no more than ±2°F in a range of 70° to 80°F and 40% to 70% humidity for rats and mice. The U.S. Department of Health, Education, and Welfare (1974) suggests levels of 65° to 85°F and 30% to 70%, depending on the test species. Sontag, Page, and Saffiotti (1976) recommend more precise levels of 74°F ± 2° and 40% ± 5% for rats and mice. In any case, the investigator should define the levels used, along with the population size and cage type, so that the study can be properly evaluated.

2.3.8 Light

Light is another environmental variable that should be controlled. The damaging effects of light are dependent on the intensity and duration (Weihe, 1976*b*). All common laboratory animals are sensitive to light, and by using low-level, artificial light sources, with exclusion from alternate sources such as windows, the proper control can be maintained (Weihe, 1976*b*).

Strong light has proved to decrease the reproductive ability of mice (Porter, 1967) and to damage the retina and indirectly the endocrine system (Fox, 1977; Weihe, 1976*b*). Therefore, the light should be diffused through the animal room, with minimum levels of 100 lux at the level of the cage racks (U.S. Department of Health, Education, and Welfare, 1974). Hurni (1970) suggests room levels of 300 to 500 lux for rodents. In the computation of light levels, the effect of opaque or translucent cage construction should be considered, since this can greatly affect the level of light reaching the test animal (Clough, 1976).

The duration of the photoperiod should also be controlled, preferably in a constant pattern (U.S. Department of Health, Education, and Welfare, 1974; Weihe. 1976*b*). The daylight cycle should be designed to suit the test species, for example, 10 h for rats and mice, but 14 h for cats (Hurni, 1970). In addition to controlling the diurnal pattern, it is necessary to standardize the time of "day" when blood samples and sacrifices are made, in order to reduce variation (Fouts, 1976).

2.3.9 Ventilation

Proper ventilation of the testing facility and particularly the animal room is necessary to maintain low concentrations of atmospheric contaminants (e.g., particulates, CO_2, NH_3, moisture, and microorganisms), reduce odors, regulate temperatures, and promote comfort (Clough, 1976; U.S. Department of Health, Education, and Welfare, 1974). The ability to limit odor depends on the species used and the population densities, as well as the proper design of the facility (U.S. Department of Health, Education, and Welfare, 1974). The precise pattern of air movements in each room results from the interaction of convection currents arising from the test animals, from the air input by the ventilation system, and from the deflections caused by the cages, racks, and equipment (Clough, 1976). Nevins (1971) discusses the design criteria needed to optimize the ventilation patterns. Rooms used by humans and animals should each be ventilated separately (National Academy of Sciences, 1969; U.S. Department of Health, Education, and Welfare, 1974). The air should be changed frequently without causing drafts, and incoming or recirculated air must be filtered (National Academy of Sciences, 1969; Hurni, 1970; Sontag, Page, and Saffiotti, 1976). The recommended rates of air changes per hour include: 6 to 15 (National Academy of Sciences, 1969); 15 to 23 (Hurni, 1970); and 10 to 15 (Shaw, 1976; Sontag, Page, and Saffiotti, 1976; U.S. Department of Health, Education, and Welfare, 1974). The exact rate will depend on the population density, refuse removal schedule, test design, and degree of desired comfort (Hurni, 1970; National Academy of Sciences, 1969). Hurni (1970) also suggests that the air should be negatively charged (about 2000 negative ions per cubic centimeter) to increase the oxidation of odors and microorganisms.

The use of special ventilation systems for testing facilities requires some specific precautions. If a "clean-dirty" corridor system is used, the air pressure in each room should be positive to the "dirty" corridor and negative to the "clean" one (Sontag, Page, and Saffiotti, 1976). This will help reduce the possibility of backflow contamination. For a laminar flow system where air is introduced at slow rates of speed from large wall-sized vents, the room design and equipment placement must be carefully considered, because turbulence and eddy effects could disrupt the flow (Shaw, 1976). Other design considerations affecting air movements and filtration are reviewed by Shaw (1976) and Dymet (1976).

2.3.10 Noise and Handling

Noise can be undesirable because of its effects, often underrated, on test animals and personnel. Because there is always some unavoidable background noise, it should be considered in designing test facilities and toxicity studies (Hurni, 1970; U.S. Department of Health, Education, and Welfare, 1974). The sources of noise are derived from the feeding and cleaning operations, ventilation equipment, animal vocalizations (especially dogs and monkeys), and animal/cage contacts (Fletcher, 1976). The damaging effects of noise include the expected auditory (damage to ear structures and tissues) and nonauditory (stress) effects, which have been demonstrated in special acute studies (Fletcher, 1976). However, extrapolation of these effects to chronic laboratory situations is uncertain because many critical factors such as species auditory levels and frequency, intensity, and temporal patterns of common noises are unknown. In general, effects of noise should be minimized, especially where the exposure could be lengthy or where studies are very delicate (Fletcher, 1976). Noise can be minimized by the separation of animal and human occupancy zones, use of soundproofing materials, padding of equipment, proper personnel training, transferring cleaning chores outside the housing area, and acclimating the animals to unavoidable background noises (Fletcher, 1976; Hurni, 1970; U.S. Department of Health, Education, and Welfare, 1974). If these procedures are followed, the effect of noise on the test results should be insignificant.

Handling, or contact between the laboratory personnel and the test animals, can be a detrimental factor that reduces the standardization of environmental factors (Hurni, 1970). Improper handling may produce unnecessary stress or injury to the test animal or personnel (Short, 1967). Porter (1967) found that increased handling of mice affected their reproduction and lactation and caused some reduction in weight gain. On the other hand, proper handling produces tameness, which is healthy for the animal and expedient for the researcher. However, even with increased training of personnel and the use of the proper techniques, such as those discussed by Short (1967), the chance for differential treatment exists and should be considered, especially for comparative studies (Hurni, 1970).

2.3.11 Personnel

One of the basic factors in the design of a successful husbandry program is the selection and training of staff personnel. The numbers and types of personnel utilized and the chain of command developed depend on the goals and role of each toxicity testing facility (U.S. Department of Health, Education, and Welfare, 1974). Lane-Petter (1967) outlines an example of one such staff setup. However, there are certain standards that all staffs and personnel must meet. Basically the staff must include animal technicians (usually at least junior and senior technicians; the number of levels depends on the size of facility) and veterinary personnel (as supervisors or consultants) (National Academy of Sciences, 1969). The personnel caring for and monitoring health of the animals must be trained in the theoretical and practical aspects of their jobs (Fox, 1977). Usually the theoretical or course work requires certification by such organizations as the American Association of Laboratory Animal Science (Fox, 1977) or the Canadian Association for Laboratory Science Program (Arnold et al., 1977). Typically these organizations certify personnel on three levels: assistant animal technician, animal technician, and animal technologist. The individual facility can then provide the practical onsite training, which ensures the necessary training flexibility (Fox, 1977). To assist the veterinarian or toxicologist in the treatment, test procedures, and assessment roles, veterinary technologists or toxicology technologists are often employed (Fox, 1977). By the use of such certified and special personnel, many of the problems associated with husbandry requirements can be minimized so that the test results are not affected.

2.3.12 Conclusions

Many husbandry factors contribute to a scientifically proper toxicity evaluation. The selection of test animals should result in subjects with standardized life cycle variables, uniform genetic composition, and, in some cases, controlled levels of health (e.g., SPF strains). After selection, the animals are usually transported and should be given time to acclimate before testing. They also should be quarantined to prevent introduction of disease into the testing facility.

The quarantine is part of a larger disease-control program based on proper planning and thorough sanitary maintenance procedures. The design of the testing facility is another variable that can contribute to the success of a toxicity evaluation. A proper testing facility should include separate human and animal areas, specialized laboratories for maintenance and assessment of the animals, a supply-receiving area, a quarantine area, and an incinerator. The design should also provide for proper environmental controls. The cage design, especially solid plastic models, affects the microclimate and thereby the toxicity potential of the test chemical. Bedding material can be another source of nontreatment toxicity and should not be composed of fresh softwoods or hardwoods with high tannic acid content. The temperature and humidity of the testing environment should be controlled within a range of 65° to 85°F and 30% to 70%, depending on the species, with only gradual changes. The available light should be regulated to provide diffuse levels with a standard photoperiod duration. Ventilation and filtration of the housing atmosphere can control many other environmental factors and is often designed to reduce contamination factors (e.g., "clean-dirty" corridor system). Noise and handling variables can increase animal stress and may invalidate the test results. The correct combination of well-trained personnel likewise minimizes test interference. Only by control of all these husbandry variables will the test results accurately reflect the toxicity of the test chemical.

2.4 DIET

2.4.1 Introduction

The major issues necessary to the discussion of diets of laboratory animals include general nutritional adequacy of diets, types of diets available (commercial, open-formula, semisynthetic), and the presence in the diet of toxic contaminants. Also important, in the context of this document, are the effects of diet on the response of experimental animals to toxic substances.

2.4.2 Dietary Requirements for Laboratory Animals

Basic dietary requirements are similar for various common laboratory animal species (Clarke et al., 1977). All require proteins, carbohydrates, fats, minerals, micronutrients, and energy.

Dietary proteins are the source of the amino acids that an animal requires to build its own proteins. The protein content of the diet should ideally contain, in the correct proportions, all the "essential" amino acids (those not formed at all in the animal's own tissues or not formed at a rate fast enough to satisfy demand) (Clarke et al., 1977). Table 2.3 is a summary of the attempts of various investigators to determine the relative proportions in which the essential amino acids are required by the growing rat (Coates et al., 1969). The composition of two proteins, hen's egg and human milk, are also presented. These are both known to be 100 percent utilizable by the rat and are assumed to have practically ideal proportions of the essential amino acids. Since the amino acid requirements for mice and rats seem to be similar a single "target pattern," given in the last column of Table 2.3, is proposed for both species. Clarke et al. (1977) suggested that the same target pattern would be satisfactory for all common laboratory animals.

Carbohydrates are the major energy source in most diets for laboratory animals. It has been shown that energy requirements for maintenance are closely related to basic metabolic rate; therefore, the requirement for energy is higher during pregnancy, lactation, and periods of rapid growth.

Lipids in the diets of laboratory animals are also a source of energy and are necessary for utilization of fat-soluble vitamins. Essential fatty acids are required for synthesis of tissue and cell components.

Other general dietary requirements for laboratory animals include vitamins A, D, E, K, B, and C and choline. Essential inorganic elements or minerals include electrolytes (sodium, potassium, calcium, magnesium, chloride, and phosphate), which contribute to ionic and osmotic balance between cells, tissue fluids, and plasma. Minor and trace elements include iron, copper, zinc, magnesium, iodine, cobalt, manganese, and selenium, which take part in intracellular metabolic processes as metalloprotein complexes and coenzymes.

Table 2.3. Requirements for essential amino acids expressed as percentage of protein

	Rat								Mouse		Human milk	Whole egg	"Target pattern"
	Reference[a]												
	1	2	3	4	5	6	7	8	9	10	11	11	
Arginine		5.0	9.0		4.6			1.7		1.9	4.0	6.6	5.0
Histidine	4.0	2.5	2.9	1.8	2.0	2.0		2.5		3.4	2.2	2.4	2.5
Lysine	10.1	9.0	11.8	5.2	3.7	6.5		7.5		9.0	6.4	6.4	6.0
Tyrosine		3.0			4.7					4.8	5.7	4.2	4.0
Tryptophan	1.0	1.1	1.0	1.0	1.5	1.3		1.3		1.9	1.7	1.6	1.5
Phenylalanine	4.6	4.2	7.7	4.9	6.3			7.5		14.[illegible]	4.8	5.8	5.0
Methionine + cystine	7.5	5.0	5.4	4.7	4.4		4.6	5.0	3.0	8.0	4.4	5.5	4.5
Threonine	5.0	5.0	3.5	4.1	2.6	4.2		4.2		6.8	4.6	5.1	4.0
Leucine	8.0	7.0	7.3	7.8	9.5	8.2		6.7		12.5	9.2	8.8	8.0
Isoleucine	5.0	5.5	4.8	4.3	5.1	5.0		4.2		8.7	6.6	6.6	5.0
Valine	7.0	5.5	5.4	5.0	6.2			5.8		9.7	6.7	7.3	5.5

[a] Ref. 1 – Rose, W. C., M. J. Oesterling, and M. Womack. 1948. Comparative Growth on Diets Containing Ten and Nineteen Amino Acids with Further Observations upon the Role of Glutamic and Aspartic Acids. J. Biol. Chem. 176:753. Ref. 2 – Rama Rao, P. B., V. C. Metta, and B. C. Johnson. 1959. The Amino Acid Composition and the Nutritive Value of Proteins. I. Essential Amino Acid Requirements of the Growing Rat. J. Nutr. 69:387. Ref. 3 – Rogers, Q. R., and A. E. Harper. 1964. Amino Acid Diet for Rats. Fed. Proc. Fed. Am. Soc. Exp. Biol. 23:186. Ref. 4 – Bender, A. E. 1961. Determination of the Nutritive Value of Proteins by Chemical Analysis. In: Progress in Meeting Protein Needs of Infants and Preschool Children. Publ. Natl. Res. Coun., Wash. 843:407. Ref. 5 – Fisher, R. B. 1954. Protein Metabolism. London: Methuen. Ref. 6 – McLaughlin, J. M., and W. I. Illman. 1967. Use of Free Plasma Amino Acid Levels for Estimating Amino Acid Requirements for the Growing Rats. J. Nutr. 93:21. Ref. 7 – Hartsook, W. E., and H. H. Mitchell. 1956. The Effect of Age on the Protein and Methionine Requirement of the Rat. J. Nutr. 60:173. Ref. 8 – National Research Council. 1962. Nutritional Requirements of Domestic Animals, Number 10: Nutritional Requirements of Laboratory Animals. National Research Council, Washington. 990. Ref. 9 – Leveille, G. A., H. E. Sauberlich, and J. W. Shockley. 1961. Sulfur Amino Acid Requirements for Growth of Mice Fed Two Levels of Nitrogen. J. Nutr. 75:455. Ref. 10 – Bauer, C. D., and C. P. Berg. 1943. The Amino Acids Required for Growth in Mice and the Availability of the Optical Isomers. J. Nutr. 26:51. Ref. 11 – FAO. 1965. Protein Requirements. FAO Nutr. Mtg. Rep. Ser. 37.

Source: Coates et al., 1969.

In selecting the proper diet the investigator must bear in mind that basal dietary requirements, as well as specific nutritional requirements of animals, may vary with species or strain and with age, sex, gestation, lactation, disease, season of the year, temperature, relative humidity, ventilation, unknown internal physiological mechanisms, and stress (of experimentation) (Hughes and Lang, 1978). Specific requirements for the various species of laboratory animals are discussed in detail by Clarke et al. (1977) and in the National Academy of Sciences – National Research Council (1972) document *Nutrient Requirements of Laboratory Animals*.

2.4.3 Types of Diets for Laboratory Animals

In choosing a feed one should consider: (1) constancy of its major ingredients and their sources; (2) timely delivery; (3) moisture content; (4) freshness; (5) storage characteristics (Sontag, Page, and Saffiotti, 1976); and (6) purity.

Many commerical diets are available for laboratory animals. The cost of a diet is a small part of the overall cost of bioassays, and the investigator should buy the best diet to ensure long-term survival under optimal nutritional conditions (Sontag, Page, and Saffiotti, 1976; Weisburger, 1976).

The goal in testing must be to standardize the diet in and among animal studies so that data can accurately be compared on a worldwide level. Therefore, in the formulation of a standard diet international availability of the ingredients should be considered.

The Food Safety Council (1978) recommends the use of standardized diets in accordance with guidelines of the National Academy of Sciences' 1972 Subcommittee on Laboratory Animal Nutrition and 1978 Committee on Laboratory Animal Diets.

Laboratory animal diets have been characterized by the American Institute of Nutrition (1977) as:

Cereal-based diet Unrefined diet Nonpurified diet	Diet formulations composed predominantly of unrefined plant and animal materials that may contain added vitamins or minerals – stock diet, laboratory chow, etc.

Open-formula	Diet in which precise percentage composition of each ingredient is available either in the published scientific literature or in available commercial information. No changes are permitted in type or amount of ingredients.
Closed-formula	Diet in which exact composition by type and amount of each ingredient is not disclosed by the manufacturer. Combination of ingredients may change with market conditions.
Purified diet	Diets composed primarily of refined ingredients such as commercially refined proteins, carbohydrates, and fats with vitamins and minerals added. (Semisynthetic.)
Chemically defined diet	Diets characterized by the nitrogen source being provided by pure amino acids, carbohydrates from refined mono- or disaccharides, and fats from purified fatty acids or triglycerides. Minerals are reagent grade, and vitamins are of high purity.

Use of open-formula diets is encouraged by the Food Safety Council (1978) and the American Institute of Nutrition (1977) and in *Guidelines for Carcinogen Bioassay in Small Rodents* (Sontag, Page, and Saffiotti, 1976).

The open-formula, cereal-based diet (NIH-07) proposed by the American Institute of Nutrition (1978) was developed by the National Institute of Health in 1972 and is shown in Table 2.4. The NIH-07 open-formula rat and mouse diet has been found to be satisfactory for reproduction, lactation, and maintenance of the animals. It is produced by several feed manufacturers and is priced competitively with commercial diets (American Institute of Nutrition, 1977). In spite of high cost, the semisynthetic diet may be preferred for some studies (Page, 1977). For example, practical experience has shown that semisynthetic diets could be of use in subchronic studies, although they have not been particularly satisfactory in chronic reproduction studies (Food Safety Council, 1978).

The purified diet AIN-76® and the vitamin and mineral mixtures used in this diet are shown in Tables 2.5, 2.6, and 2.7. This diet supports growth, lactation, and reproduction in rats and mice comparable to the NIH-07 diet (American Institute of Nutrition, 1977).

Table 2.4. NIH-7 open-formula rat and mouse ration[a]

Ingredient	%
Dried skim milk	5.00
Fish meal (60% protein)	10.00
Soybean meal (49% protein)	12.00
Alfalfa meal (dehydrated, 17% protein)	4.00
Corn gluten meal (60% protein)	3.00
Ground No. 2 yellow shelled corn	24.50
Ground hard winter wheat	23.00
Wheat middlings	10.00
Brewer's dried yeast	2.00
Dry molasses	1.50
Soybean oil	2.50
Sodium chloride	0.50
Dicalcium phosphate	1.25
Ground limestone	0.50
Premixes[b]	0.25
Total	100.00

[a] Calculated proximate composition — crude protein, 23.5%; crude fat, 5.0%; crude fiber, 4.5%; ash, 7.0%.

[b] Vitamin and mineral premixes shall provide per kilogram of diet: vitamin A (stabilized), 6050 IU; vitamin D_3, 5060 IU; vitamin K, 3.1 mg; α-tocopheryl acetate, 22 IU; choline 0.6 g; folic acid, 2.4 mg; niacin, 33 mg; d-pantothenic acid, 20 mg; riboflavin, 3.7 mg; thiamin, 11 mg; vitamin B-12, 4.4 μg; pyridoxine, 1.9 mg; biotin, 0.15 mg; cobalt, 0.44 mg; copper, 4.4 mg; iron, 132 mg; manganese, 66 mg; zinc, 18 mg; iodine, 1.5 mg.

Source: American Institute of Nutrition, 1977. Reprinted with permission of the publisher.

For special circumstances, controlled variation of experimental diets to simulate the variety of human diets has been suggested by the World Health Organization (1978) and the National Academy of Sciences (1975). For example, animal diets could be shifted to a higher fat content to allow for the expression of some cocarcinogenicity of fats or to a lower protein content to simulate human deficiency states.

Regardless of the type of diet chosen, all test animals and their corresponding controls should be maintained on identical diets, and when

Table 2.5. AIN-76® purified diet[a,b] (for rats and mice)

Ingredient	%
Casein[c]	20.0
DL-Methionine	0.3
Cornstarch	15.0
Sucrose	50.0
Fiber[d]	5.0
Corn oil[e]	5.0
AIN mineral mix[f]	3.5
AIN vitamin mix	1.0
Choline bitartrate	0.2
Total	100.0

[a] Trademark pending.

[b] This diet is intended for growth and maintenance during the first year of life. Investigators should be aware that diets high in sucrose can be cariogenic and that some strains of rats fed such diets may develop kidney lesions after extended periods. The diet has been found to be satisfactory for reproduction and lactation in both rats and mice. If used for deficiency studies, modifications will be necessary. If used in ultraclean environment, several trace elements should be added (Federation Proc. 33, 1748-1773, 1974). The diet can be pelleted satisfactorily, if desired, by addition of water (no binder).

[c] Feed-grade casein having at least 85% protein.

[d] Cellulose-type fiber.

[e] Some commercial corn oils contain antioxidants (maximum 0.02%) and a surfactant (dimethyl silicone). These additives should be innocuous for most nutritional studies, but investigators should be aware of their presence. It is recommended that an oil with added antioxidant be used to prevent rancidity. Diet should be stored at 4°C or colder, and it is recommended that the diet not be kept longer than 4 months.

[f] The total dietary content of some minerals, due to their presence in casein, will be slightly higher and will vary according to the casein used.

Source: American Institute of Nutrition, 1977. Reprinted with permission of the publisher.

Table 2.6. AIN-76® vitamin mixture[a]

Vitamin	Per kilogram of mixture
Thiamin•HCl	600 mg
Riboflavin	600 mg
Pyridoxine•HCl	700 mg
Nicotinic acid[b]	3 g
D-Calcium pantothenate	1.6 g
Folic acid	200 mg
D-Biotin	20 mg
Cyanocobalamin (vitamin B-12)	1 mg
Retinyl palmitate or acetate (vitamin A)	+[c]
dl-α-Tocopheryl acetate (vitamin E)	+[d]
Cholecalciferol (vitamin D_3)	2.5 mg[e]
Menaquinone (vitamin K)[f]	5.0 mg
Sucrose, finely powdered	To make 1000.0 g

[a] Based on the National Academy of Sciences-National Research Council recommended levels for rats (National Academy of Sciences-National Research Council, 1972). To be used at 1% of diet.

[b] Nicotinamide is equivalent.

[c] As stabilized powder to provide 400 000 IU vitamin A activity or 120 000 retinol equivalents.

[d] As stabilized powder to provide 5000 IU vitamin E activity.

[e] 100 000 IU. May be in powder form.

[f] Menadione.

Source: American Institute of Nutrition, 1977. Reprinted with permission of the publisher.

a change in diet manufacture occurs, all animals should be changed to the new diet at the same time (Sontag, Page, and Saffiotti, 1976; Weisburger, 1976). The diets should be well tried and readily available to the animals (Magee, 1970).

In addition, feed must be protected from air, light, heat, chemical fumigants, radiation, and contamination by microorganisms — all of which may damage the nutrient quality of a diet (Clarke et al., 1977). Sontag,

Table 2.7. AIN-76® mineral mixture[a]

Ingredient	Grams per kilogram of mixture
Calcium phosphate, dibasic ($CaHPO_4$)	500.0
Sodium chloride ($NaCl$)	74.0
Potassium citrate, monohydrate ($K_3C_6H_5O_7 \cdot H_2O$)	220.0
Potassium sulfate (K_2SO_4)	52.0
Magnesium oxide (MgO)	24.0
Manganous carbonate (43% to 48% Mn)	3.5
Ferric citrate (16% to 17% Fe)	6.0
Zinc carbonate (70% ZnO)	1.6
Cupric carbonate (53% to 55% Cu)	0.3
Potassium iodate (KIO_3)	0.01
Sodium selenite ($Na_2SeO_3 \cdot 5H_2O$)	0.01
Chromium potassium sulfate [$CrK(SO_4)_2 \cdot 12H_2O$]	0.55
Sucrose, finely powdered	To make 1000.0

[a] Based on the National Academy of Sciences-National Research Council requirements for rats (National Academy of Sciences-National Research Council, 1972). To be used at 3.5% of the diet.

Source: American Institute of Nutrition, 1977. Reprinted with permission of the publisher.

Page, and Saffiotti (1976) recommend sterilization of feed (without degrading nutrients) when practical and consistent with the disease control program of the laboratory.

2.4.4 Analysis for Nutrients and Contaminants

Contaminants in the diet and variations in the concentration of essential nutrients can influence the response of animals in toxicity tests and thus alter the interpretation of experimental data (Food Safety Council, 1978; Fox, 1977; Newberne, 1975).

Variations in the concentration of essential nutrients can occur for many reasons: batch or lot differences, regional and seasonal differences, manufacturer variations, and processing. Most diets contain nutrients in quantities sufficient for the growth, maintenance, and reproduction of a particular species. However, concentrations of essential ingredients may vary from batch to batch of a formulation made with different lots of natural ingredients, and at the same time the guaranteed analysis shown on the label can remain correct (Newberne, 1975).

Differences in manufacturing procedures were pointed out by Porter, Lane-Petter, and Horne (1963), who compared growth rates of groups of young mice fed diets produced in the same region by three different manufacturers and found considerable differences (cited by Hurni, 1970). Also, nutritional quality may be seriously impaired during processing, particularly by overheating, which can destroy amino acids or lead to the formation of indigestible complexes (Clarke et al., 1977).

These examples help to illustrate the need for periodic analysis of diets for nutrient content, as proposed by the National Research Council (1977).

Because of the presence of a variety of contaminants in animal diets it is also advisable to test periodically for pesticides, mycotoxins, trace minerals, and industrial contaminants (such as polychlorinated biphenyls, lead, and mercury) (Food Safety Council, 1978; National Research Council, 1977; Newberne, 1975; Page, 1977). Low levels of *N*-nitrosamines have been detected in the diets of experimental animals in Germany and in the United States (Edwards et al., 1979; Walker, Castegnaro, and Griciute, 1979). The source of the nitrosamines is thought to be fishmeal, and Knapka (1979) suggested that the *N*-nitrosamines are present only in diets purchased in meal form.

There is a growing interest in obtaining detailed open-formula information for assurance that diets are free from pesticide residues and other contaminants (Food Safety Council, 1978); if prospective analysis for contaminants cannot be arranged with feed suppliers, retrospective analysis could be conducted.

The ultimate solution to the problems of uniform constituents and toxic contaminants in animal diets may be provided by the use of rigorously purified semisynthetic diets or entirely synthetic diets (Clarke et al., 1977; Food Safety Council, 1978).

2.4.5 Effects of Diet on Toxicity Test Results

Nutritional imbalances may influence toxicity through physiological, immunological, and/or biochemical mechanisms (Campbell and Hayes, 1974). The main effects, however, can be attributed to the effects of nutritional state on the drug-metabolizing enzymes located in the endoplasmic reticulum of the hepatocyte. This enzyme complex, classified as a mixed-function oxidase, is composed of cytochrome P-450, phosphatidycholine, and a flavoprotein reductase. A wide variety of drugs and foreign compounds are metabolized by the complex to products of greater or lesser toxicity. The response of the mixed-function oxidase system to toxic substances can be modified by deficiency or excess of dietary constituents such as protein, carbohydrates, fats, lipotropes, vitamins, and minerals. Examples of these effects in animals will be reviewed in the following sections.

2.4.5.1 Protein The dietary constituent most studied is protein. Qualitative and quantitative changes in protein content can alter mixed-function oxidase activities. McLean and McLean (1969) pointed out opposite effects of protein deficiency on the toxicity of compounds that are detoxified and the toxicity of those that are rendered toxic by biotransformation. For example, protein-deficient diets protect against acute poisoning from carbon tetrachloride and dimethylnitrosamine in rats (McLean and McLean, 1966; McLean and Verschuuren, 1969), whereas the toxicities of most pesticides are increased during protein deficiency (Boyd, 1969). Protein-deficient rats have become more susceptible to the toxicity of aflatoxin when administered alone and of chloroform following enzyme induction with DDT; but chloroform toxicity in a single oral dose is not altered by protein deficiency (Madhavan and Gopalan, 1965; McLean and McLean, 1969). Severe protein depletion has been implicated in the production of increased resistance to mercuric chloride poisoning in rats (Surthin and Yagi, 1958).

Metabolism of a number of carcinogens is affected by dietary protein content. The carcinogenic action of dimethylnitrosamine has been enhanced in rats by protein deficiency leading to suppression of microsomal hydroxylation (Swan and McLean, 1968).

Silverstone and Tannenbaum (1951) demonstrated that the formation of spontaneous hepatomas was substantially retarded by a decrease (from 18% to 9%) in dietary protein, while increased protein intake (from 18% to 45%) had no effect on tumor formation. The inhibition was found to be due to a deficiency in sulfur-containing amino acids in the diet, not to the difference in the proportion of total protein. Kawachi, Hirata, and Sugimura (1968) demonstrated that addition of 1% tryptophan to the diets of rats receiving a low level of *N*-nitrosodiethylamine increased the liver cancer incidence almost fourfold. Bryan, Brown, and Price (1964) reported that *L*-tryptophan enhanced the induction of bladder tumors by 2-acetamidofluorene.

In other studies, deficiencies of protein and individual amino acids have been related to congenital malformations in rats. Early experiments in reproduction and embryogenesis were reviewed by Kalter and Warkany (1959).

2.4.5.2 Lipids — Lipids provide energy and essential fatty acids for synthesis of tissue and cell components. High fat diets sensitize animals to the toxic effects of chloroform (Goldschmidt, Vars, and Raudin, 1939).

Marshall and McLean (1969) showed that dietary addition of either herring oil, linoleic acid, or 0.1% oxidized sitosterol was required for maximum induction of cytochrome P-450 synthesis.

Rogers et al. (1974) and Rogers (1975) found that liver tumor induction with various carcinogens was enhanced if the diet contained a high fat content and was deficient in choline, methionine, and folic acid. Carroll and Khor (1970) reported that Sprague-Dawley rats on a high fat diet developed more mammary tumors after administration of 7,12-dimethylbenzanthracene than did rats on a low fat diet.

2.4.5.3 Lipotropes — Lipotropes are required for the synthesis of phospholipids, which transport triglycerides. Lipotrope deficiency is characterized by accumulation of triglycerides in the liver. Aflatoxin has an increased hepatotoxic effect in rats on a low lipotrope diet (Newberne, Rogers, and Wogan, 1968). Newberne and Rogers (1976) reported a higher incidence of *N*-2-fluorenylacetamide- and dimethylbenzanthracene-induced mammary tumors in Sprague-Dawley rats on a low lipotrope, high fat diet than in animals on a regular diet. Lipotrope-deficient Fischer rats, on the other hand, which were resistant to mammary tumor induction with *N*-2-fluorenylacetamide, developed a significantly higher number of hepatic carcinomas than did controls.

2.4.5.4 Vitamins — The role of vitamin deficiencies in the mixed-function oxidase system has been reviewed comprehensively by Campbell and Hayes (1974). The effects do not appear to be as serious as the effects of protein deficiencies, although activities are affected to some degree if the vitamin deficiencies are severe enough. The influence of vitamins on the production of congenital malformations has been reviewed by Kalter and Warkany (1959).

Reports of the effects of vitamin A on experimental tumor induction are conflicting. Saffiotti et al. (1967) and Cone and Nettesheim (1973) demonstrated inhibition by vitamin A of the induction of squamous metaplasia and squamous cell tumors of the respiratory tract of rats and hamsters with polycyclic hydrocarbons. However, Smith et al. (1975) and Smith, Rogers, and Newberne (1975) reported enhancement by vitamin A of the induction of respiratory tract tumors by benzo(*a*)pyrene.

2.4.5.5 Minerals — Deficiencies in dietary trace minerals, such as iron, iodine, zinc, selenium, and copper, and their effects on the mixed-function oxidase system have been reviewed by Campbell and Hayes (1974). Moffitt and Murphy (1974) have shown that the trace mineral concentration can influence the metabolism, distribution, and toxic action of chemicals. The interactions between nutritional minerals and toxic substances may be of a deleterious or protective nature (Brinkman and Miller, 1961; Farkas, 1978). For example, Brinkman and Miller observed that toxicity to molybdnenum was increased in rats whose diets contained higher amounts of zinc than usual. Farkas, however, suggested that selenium may play a role in decreasing the toxicity of cadmium and mercury through its interactions with those metals.

2.4.6 Conclusions

Diets of experimental animals should contain proteins, carbohydrates, fats, minerals, micronutrients, and energy in the proportions suitable for a particular species or strain. Various types of diets available include nonpurified or cereal based, open-formula, closed-formula, purified or semisynthetic, and chemically defined.

There is growing concern regarding contaminants in the diet and variations in the concentrations of essential nutrients and their consequent effects on the response of test animals to toxic substances. Periodic testing of diets for nutrient concentration and contaminants has been proposed, but the ultimate solution to the problem may be provided by the use of synthetic or semisynthetic diets.

2.5 PATHOLOGY

2.5.1 Introduction

The importance of proper pathology examination and reporting techniques cannot be overemphasized (Prieur et al., 1973). They must be planned before the start of the experiment because they influence the test design and the selection of the animal models (Page, 1977). Quality control must be emphasized at all times, because important lesions may be lost at any of several steps: necropsy, trimming, fixation, paraffin blocking, or slide preparation (Page, 1977). With the proper techniques and quality control, a pathology workup gives information on the morphology of the chemically induced lesions present at that time and some indication of a dose-effect relationship (Zbinden, 1976). However, the limitations of pathology must be recognized, including a lack of information on early lesions that disappear before necropsy, the sequence of lesion appearance, the reversibility of lesions, and the developmental history (morphogenesis) of each lesion (Zbinden, 1976). The following subsections will discuss the procedures that should be used to obtain the desired pathology information.

2.5.2 Gross Examination

The gross examination or necropsy is the first step in a pathology evaluation and the most influential factor in determining the toxic effects of a chemical. Unless it is done properly, much valuable information will be lost. Therefore the necropsy should either be performed directly by the pathologist (or the scientist who is to do the microscopic examination) or by a trained technician with consultation from the pathologist (Food Safety Council, 1978; Magee, 1970; Prieur et al., 1973; Sontag, Page, and Saffiotti, 1976; U.S. Food and Drug Administration, 1971; World Health Organization, 1978). An extensive necropsy (including blood counts, bone marrow smears, and organ weights) is a desirable objective, but usually the design must be varied for each experiment (Roe, 1965).

To ensure that the maximum information is obtained from the test animals, a daily observation should be included to reduce loss from postmortem degeneration (Magee, 1970; Roe, 1965). The necropsy should be done as soon as possible after death and, if not, the animal should be placed in a refrigerator for storage (Prieur et al., 1973). Even if some tissue degeneration has occurred, a necropsy should be performed, because it can indicate the general lesions to be expected (Magee, 1970; World Health Organization, 1978). If an animal is moribund, sacrifice is often required and should be determined based on a standard set of criteria or on the experience of the observer (Magee, 1970). However, the number of animals sacrificed should not exceed the short-term capacity of the examiners (World Health Organization, 1978).

The organs examined should all be identified regardless of the results (Roe, 1965). It is often easiest and most thorough to use a checklist system (U.S. Food and Drug Administration, 1971; World Health Organization, 1978). The examination of each organ should be performed in a standard procedure, with the same slices and angles examined for every test animal (Roe, 1965). During the examination, all gross lesions observed should be recorded and described, including information on the size, location, number, shape, color, and texture (Sontag, Page, and Saffiotti, 1976; World Health Organization, 1978). The weight of the organ can also be determined during a gross examination, but the results are of doubtful significance for lifetime studies (Magee, 1970). If weight is to be determined, it should be done as soon as possible to reduce drying effects (World Health Organization, 1978).

The necropsy should start with an external examination including orifices (Sontag, Page, and Saffiotti, 1976; World Health Organization, 1978). Next, examine all the internal cavities and organs in situ and do not separate the connective tissue until it also has been examined (Prieur et al., 1973). Table 2.8 lists some tissues that have been suggested for examination. Special procedures for individual organs have also been recommended: (1) parenchymal and endocrine glands should be thoroughly examined, including multiple cut slices, since these tissues often contain deep-seated lesions (Sontag, Page, and Saffiotti, 1976; Food Safety Council, 1978); (2) all hollow organs, including the urinary bladder, gastrointestinal tract, and respiratory tract, should be cut open and extensive examination of the mucosal surfaces performed (Food Safety Council, 1978; Sontag, Page, and Saffiotti, 1976; World Health Organization, 1978); (3) the skull and brain should be cut open and examined, including examination of the pituitary (Magee, 1970); and (4)

Table 2.8. Tissues to be included in a gross examination

Gross lesions
Tissue masses or suspect tumors and regional lymph nodes
Skin
Mandibular lymph node
Mammary gland
Salivary gland
Larynx
Trachea
Cecum
Colon
Rectum
Mesenteric lymph node
Liver
Thigh muscle
Sciatic nerve
Sternebrae, vertebrae, or femur (plus marrow)
Costochondral junction, rib
Thymus
Gallbladder
Pancreas
Lungs and bronchi
Heart
Thyroids
Parathyroids
Esophagus
Stomach
Duodenum
Jejunum
Ileum
Spleen
Kidneys
Adrenals
Bladder
Seminal vesicles
Prostate
Testes
Ovaries
Uterus
Nasal cavity
Brain
Pituitary
Eyes
Spinal cord

Source: Adapted from Sontag, Page, and Saffiotti, 1976.

the spinal cord in rodents is best examined in situ (World Health Organization, 1978) and often is not routinely examined (Magee, 1970).

In carcinogenicity studies, the gross examination is even more important than usual because much of the lesion incidence data are generated during this procedure (Food Safety Council, 1978). To evaluate the effectiveness of the necropsy to detect lesions, Frith et al. (1979) and Kulwich et al. (1980) correlated observations of the gross and microscopic examinations in carcinogenicity studies. Table 2.9 shows how frequently the gross examination detected lesions also seen in microscopic examinations in mice. Frith et al. (1979) concluded that the size of the organ, the stage and nature of the lesion, and the number of slices examined all affect the efficiency of the gross examination. Kulwich et al. (1980) found correlation rates between gross and microscopic examinations of 70% and 76% for two chemicals in rats. They concluded that gross examination is less successful for small organs, because a single histological slice represents a larger portion of the organ and can often find lesions not seen grossly. However, for tissues like the skin and mammary gland, the gross examination is much more successful. They also found that the size and nature of the lesions affected the gross detection.

Table 2.9. Correlation between gross and microscopic lesions in carcinogenic studies in mice

Organ	Lesions seen in gross exam	Lesions seen microscopically	Percentage found in gross
Liver	5 550	5 968	93
Thymus	725	1 480	49
Spleen	6 589	8 340	79
Mammary tissue	6 503	6 993	93
Uterus (adenocarcinomas)	50	81	62
Uterus (polyps)	2 423	6 922	35
Lung	4 503	10 007	45
Adrenal gland	711	1 776	40
Harderian gland	1 438	4 637	31
Testes	330	402	82
Pituitary gland	486	836	58

Source: Adapted from Frith et al., 1979.

2.5.3 Tissue Preservation and Storage

After the gross examination, tissues should be excised and preserved to prevent further degeneration. This should be done as quickly as possible, since degeneration can occur quite rapidly, especially with mice (Magee, 1970). Several techniques for preservation are available, with immersion being the most common (World Health Organization, 1978). However, for some tissues (lung and central nervous system) and for electron microscopy, perfusion is preferred. This involves draining the blood, followed by injection of a proper fixative (World Health Organization, 1978). Perfusion cannot be used if organ weights are needed (World Health Organization, 1978).

The fixative most recommended is neutral buffered formalin at concentrations of 10% (Prieur et al., 1973; Sontag, Page, and Saffiotti, 1976; World Health Organization, 1978) or 4% (Zbinden, 1976). Special preservatives are often required (e.g., Bouin's solution for paraffin-embedded material) depending on the stain to be used and the tissue involved (Prieur et al., 1973; Zbinden, 1976). The tissues should be preserved at a maximum thickness of 0.5 cm (Prieur et al., 1973; Sontag, Page, and Saffiotti, 1976; World Health Organization, 1978). Some tissues, like the testicles, should first be immersed intact and then sliced in 0.5-cm sections for further preservation (Zbinden, 1976). The tissue/preservative ratio on a volume basis should be greater than 1:10 (World Health Organization, 1978) and preferably 1:15 to 1:20 (Prieur et al., 1973). The tissues should be left in the preservative for at least 24 h, and up to 72 h has been suggested (Prieur et al., 1973; Sontag, Page, and Saffiotti, 1976; World Health Organization, 1978). Often special procedures are suggested, including: covering tissues that float with absorbant material (e.g., cheesecloth) for uniform preservation (Prieur et al., 1973); skin, stomach mucosa, and nerves need to be straightened out and often attached to a card or filter paper to prevent crumpling (Prieur et al., 1973; World Health Organization, 1978; Zbinden, 1976); several thoracio-lumbar vertebrae should be fixed in situ with the spinal cord (Sontag, Page, and Saffiotti, 1976); urinary bladder should be filled with fixative (Zbinden, 1976); and multiple representative samples of variable tissue masses should be fixed (Sontag, Page, and Saffiotti, 1976). After preservation, the material should be stored in plastic bags or jars and kept at least until the final conclusions are made for the study (Food Safety Council, 1978).

2.5.4 Trimming, Staining, and Embedding of Tissue

The preparation of tissues for microscopic evaluation includes techniques for trimming, taining, and embedding. The tissues should be sampled from the same specific sites for each organ (Prieur et al., 1973), and the slices should be made so that the cut surfaces represent the maximum possible area for examination (World Health Organization. 1978). Often a set trimming schedule should be used. The trimming should be performed by or in the presence of the pathologist, with consideration of the gross examination observations (Sontag, Page, and Saffiotti, 1976). The tissues should be trimmed to a thickness of 2 to 3 mm for histologic processing (Sontag, Page, and Saffiotti, 1976; World Health Organization, 1978). Specific recommendations for trimming of certain organs have been made by the National Cancer Institute (Sontag, Page, and Saffiotti, 1976) and the World Health Organization (1978). These recommendations include: (1) multiple portions of large masses or tumors should be submitted with some of the normal surrounding tissue; (2) parenchymal organs (e.g., liver) should be sliced to give the maximum observable area; (3) the kidneys should be sampled through the cortex and medulla, one by a midlongitudinal section and the other by a midtransverse section; (4) the lungs should be sectioned transversely (parallel to the body axis) including the bronchi and carina; (5) at least three brain cross sections are needed, with one through the frontal cortex and basal ganglia, one through the parietal cortex and thalamus, and one through the cerebellum with the pons; (6) the hollow organs should be trimmed to include a cross-section slide from mucosa to serosa; (7) if the larnyx is to be examined, the section should also include the pharnyx; and (8) if the nasal cavity is to be examined, three transverse sections are needed.

Routinely, the trimmed slices should be stained with hematoxylin and eosin and embedded in paraffin (Zbinden, 1976). These are sliced to 4 to 6 μm for microscopic slides (Sontag, Page, and Saffiotti, 1976;

World Health Organization, 1978). The paraffin embedding can be performed using automation techniques. Often semithin slices (1 μm) are needed for such organs as the bone marrow, kidney, or endocrine glands (World Health Organization, 1978). Special stains are often employed, for example in evaluations for the presence of fats, carbohydrates, or special structures (World Health Organization, 1978; Zbinden, 1976). These can in turn necessitate special preservation techniques. Obviously, to obtain the maximum information from the ensuing microscopic evaluation, proper planning prior to the testing is necessary, so that the special techniques for tissue preservation, staining, and embedding can be coordinated.

2.5.5 Microscopic Examination

The microscopic examination of properly prepared tissue slides is usually the final step in a pathology evaluation. Although its importance is overwhelmingly accepted, the details of the design are quite variable, depending on the type of toxicity test employed. The main questions are which tissues to examine, for how many animals and levels, and which evaluation techniques to use.

Many recommendations have been made concerning the organs that should be examined. Section 4.5 and Sect. 5.6.3 review some of the specific literature discussions for individual toxicity tests. In general, the choice depends on the scope of the test and is decided by the pathologist (Magee, 1970). The primary problem is a conflict between the desire to obtain the maximum amount of scientific information, and the limits imposed by practical economic and time constraints. Therefore, many discussions recommend only vague guidelines, although others are extremely specific and encompassing (Page, 1977). As an attempt to reach a compromise solution, Zbinden (1976) recommended a priority system based on the frequency of morphological changes that are likely to occur (Table 2.10). This system addresses both the specific organs to be examined and the test animals from each dose level from which the organs are to be taken. It is obvious that, with the number of chemicals to be evaluated, the microscopic examination will have to be based on some similar type of ranking system.

Table 2.10. Organs and tissues to be examined in routine toxicity tests

First Priority: All animals including controls

Liver (f)[a]
Kidney (f)
Adrenal glands (f)
Heart (left ventricle) (f)
Spleen (f)
Thymus
Testis
Epididymis
Lung
Bone marrow
Mesenterial lymph node
All organs showing gross changes of shape, weight, color, or structure

Second Priority: High dose animals and part of controls. Medium and low dose animals only when significant changes observed in high dose group

Thyroid
Parathyroid
Pituitary gland
Salivary glands
Stomach
Duodenum
Small intestine
Large intestine
Pancreas
Ovary
Uterus
Cervix uteri
Vagina
Seminal vesicles
Prostate
Coagulating gland
Tonsils
Brain
Spinal cord
Eye
Optic nerve
Peripheral nerve
Urinary bladder
Skin
Mammary gland
Bone-cartilage
Skeletal muscle
Gallbladder

Third Priority: Organs and tissues not examined in routine experiments, unless indicated by clinical observations or motivated by scientific interest

Heart valves
Purkinje fibers
Aorta and other blood vessels
Thoracic duct
Tongue
Teeth
Lips
Gingivae
Hard palate
Nasopharynx
Larynx
Trachea
Esophagus
Vermiform appendix
Rectum
Anus
Ureter
Urethra
Oviduct
Vulva
Penis
Paraurethral and preputial glands
Pineal gland
Spinal ganglions and roots
Sympathetic ganglions and trunk
Nerve fiber endings
Meninges
External ear
Middle ear
Inner ear
Olfactory organ
Subcutaneous lymph nodes
Tendons
Adipose tissue
Intervertebral disc
Synovial membrane
Lacrimal glands

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[a] (f) — frozen sections stained for fat obligatory.

Source: Adapted from Zbinden, 1976. Reprinted by permission of the publisher.

Another approach to reducing the amount of microscopic examination has been proposed by Fears and Douglas (1977; 1978). On the basis of a statistically valid sampling method, they suggest examination of all of the treatment groups first and then limited examination of the controls. The reduction occurs by limiting examination of the control groups to tissues that were found to be affected in the treatment groups. They also suggest several further modifications of this system, which result in even less pathology. One involves random sampling of the treatment groups based on gross observations to reduce pathology in those groups. Another incorporates techniques to allow for "blind" evaluations where the potential biases of the investigator are controlled. These approaches can reduce the microscopic examination by 10% to 50%. However, Kulwich et al. (1980) and Frith et al. (1979), in their evaluations of the correlation between the gross and microscopic examinations, found that one cannot rely totally on the gross examination to select affected tissues for microscopic study. They stressed that a thorough microscopic examination is still necessary. Therefore, the approach suggested by Fears and Douglas still needs more refinement, but represents a potential solution for reducing the amount of microscopic examination.

No matter what organs are examined microscopically, the information will have to be evaluated and presented in a uniform manner. It is often necessary to use a checklist to ensure that all intended organs were examined (World Health Organization, 1978). Also, standard criteria should be developed and reported for the descriptions and classifications of the lesions, especially if these are done semiquantitatively (World Health Organization, 1978). The formation of tumor tables incorporating information on the group, organ, and sex variables for each lesion can be useful for comparing chemical effects (World Health Organization, 1978).

The manner in which the slides are examined is also an issue of debate. It has been suggested that the slides should be examined by the pathologist without knowledge of the treatment or control group represented (U.S. Food and Drug Administration, 1971). This "blind" evaluation would reduce possible biases introduced by the pathologist and produce more objective conclusions (Fears and Schneiderman, 1974, as cited in Page, 1977). However, many other reviewers stress that the danger of missing lesions or incorrectly interpreting the results far outweighs bias problems, so that the pathologist should be aware of the sample group (Roe, 1965; World Health Organization, 1978; Zbinden, 1976). The Food Safety Council (1978) suggests a compromise situation where the control groups are evaluated first and the treatment groups are done "blindly." In any case, maximum use should be made of the results of prior clinical studies and gross examinations (Food Safety Council, 1978; World Health Organization, 1978; Zbinden, 1976).

2.5.6 Conclusions

Pathology is extremely useful in toxicity testing and can provide information on the chemically induced lesions present at the time of sacrifice and any dose-effect relationship. To obtain a useful pathology evaluation several requirements must be met: (1) the pathologist should perform or supervise all steps in the protocol; (2) the gross necropsy should be extremely thorough and include every animal in the study; (3) the tissues should be properly preserved in an accepted fixative immediately after the necropsy; (4) the staining, embedding, and sectioning of the tissues must be well planned and coordinated, particularly if special stains are utilized; and (5) the microscopic examination should be as thorough as practical limitations will allow. Only if these requirements are met will the pathology contribute meaningful data for evaluations of toxicity.

2.6 PHARMACOKINETICS

2.6.1 Introduction

Pharmacokinetics is the quantitative and qualitative description of the time course of absorption, distribution, metabolism, and elimination of an agent in an intact animal (Young and Holson, 1978). The following sections will examine some of the basic concepts of pharmacokinetics and show how they can be used to supplement the routine toxicological data and aid in the hazard evaluation of chemicals.

2.6.2 Basic Concepts

Withey (1978) describes absorption, distribution, metabolism, and elimination as the four major rate processes that are involved when a substance is administered to a human or animal subject. He further describes these as follows:

1. absorption — to the bloodstream from the site of administration;
2. distribution — to organs, tissues, storage depots, and receptor sites;
3. metabolism — usually in the liver but in other organs also; and
4. elimination — mainly via the urine or feces but also via the lung, from sweat and skin, and into hair.

Gehring, Watanabe, and Blau (1976) present a modification of a graphical illustration published by Garrett in 1971 of a hydraulic system used as an analogy of a three-compartment body model portraying the general concepts of pharmacokinetics (Fig. 2.1). The amount of chemical corresponding to the original dosage in the blood before any distribution to other tissue compartments is represented by D_o, whereas the amount of chemical at any given time in the vascular compartment is represented by A_v. In both the a and b parts of Fig. 2.1, the amount of chemical in the vascular compartment (A_v) has equilibrated with the amount of the chemical in the shallow or rapidly equilibrating tissue compartment (T) but not with the amount of chemical in the deep-seated or slowly equilibrating tissue compartment (T'); $T'_{\max}$ is the maximum amount of chemical that will exist in the deep-seated tissue compartment. The amount of chemical eliminated by the lungs and urine or metabolized is represented by E and is equal to $D_o - (A_v + T + T')$. As time approaches infinity, $E \rightarrow E\infty = D_o$.

Gehring, Watanabe, and Blau (1976) define a compartment in the body as those organs, tissues, and cells for which the rates of uptake and clearance of a chemical are so similar as to not permit resolution. The rapidly equilibrating compartment can be compared to those tissues with a profuse compartment blood supply, whereas the slowly equilibrating compartment can be compared to those tissues such as fat and bone that

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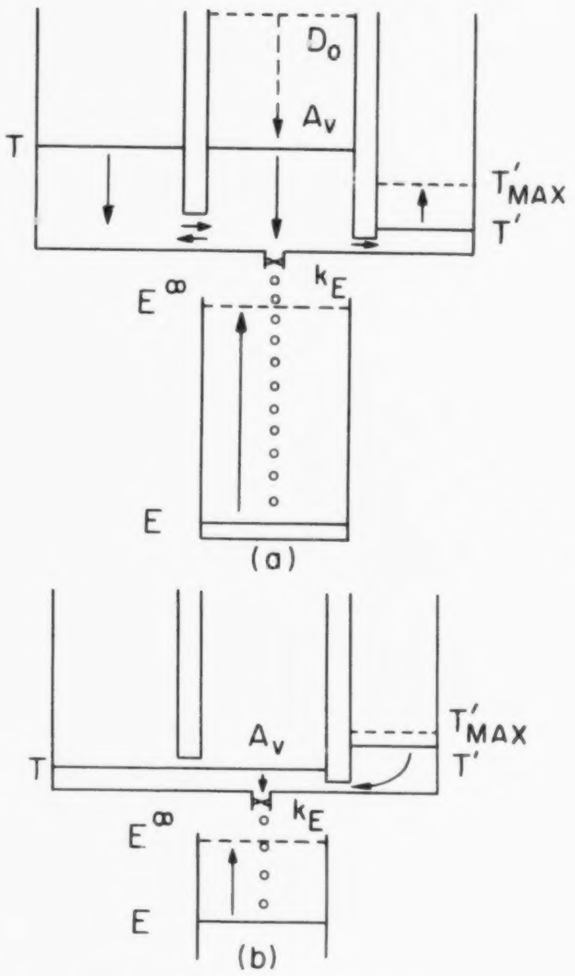

Fig. 2.1. Hydraulic analogy of three-compartment body model. D_o is the original dose in the vascular compartment prior to any equilibration at any time. A_v is the amount in the vascular compartment at any time. In both (a) and (b), A_v has equilibrated with the shallow or rapidly equilibrating compartment T. T' represents the level in the deep or slowly equilibrating compartment, and T'_{max} is the maximum level achieved in the deep compartment. E is the amount of chemical eliminated or metabolized, and $E\infty$ corresponds to the amount eliminated or metabolized at infinite time. k_e represents the overall rate constant for the elimination of the chemical from the body. Modified from Garrett (1971). Source: Gehring, Watanabe, and Blau, 1976. Reprinted with permission of the publisher.

have a more limited blood supply. Pharmacokinetic rates may be influenced by such factors as the physical characteristics of the substance (e.g., solubility, molecular weight, and surface area or solid particles), pharmacologic properties (e.g., peristalsis and blood flow), gastrointestinal functions (e.g., gastric emptying and intestinal transit and motility), permeability of membrane barriers at the site of absorption, local blood flow, metabolism, and specialized transport mechanisms (Withey, 1978).

2.6.3 Utility of Pharmacokinetics

One example of the use of pharmacokinetics as an aid in the hazard evaluation of chemicals is provided by a study of the absorption of methylcellulose (Braun, Ramsey, and Gehring, 1974). Administration of ^{14}C-labeled methylcellulose (viscosity of 3300 centipoise and molecular weight of 77 000) in both single and repeated oral daily doses of 500 mg/kg to rats showed that virtually none of the methylcellulose was absorbed, with essentially all being excreted in the feces. Therefore, if it can be demonstrated that no absorption occurs there is no need for further ingestion studies to ascertain systemic toxicity.

Another example of the utility of pharmacokinetics in the toxicological evaluation of chemicals is the oral toxicity study of 6-chloropicolinic acid (6-CPA), a degradation product of 2-chloro-6-(trichloromethyl)pyridine (CTP) (Gehring, Watanabe, and Blau, 1976). Analysis of the urine of rats given single doses of 10 mg/kg of ^{14}C-labeled 6-CPA revealed that 30% of the ^{14}C activity present was attributable to 6-CPA and 62% to the glycine conjugate. The rapid (half-life, 1.1 ± 0.2 h) and complete elimination of 6-CPA and its glycine conjugate from the rat suggested that ingestion of trace amounts of 6-CPA does not constitute a hazard. Gehring, Watanabe, and Blau state that, as expected from the pharmacological evaluation, a 90-day toxicological study in which rats were given daily doses of 6-CPA gave no indication of cumulative toxicity.

An example of the utility of pharmacokinetics to the pharmaceutical industry is given by Nelson (1976). A hypothetical situation is described of an antibiotic that is being developed for deep-seated tissue infection.

Kinetic analysis of this antibiotic in animals or human volunteers shows the drug to have a minimal distribution with a rapid elimination half-life; furthermore, renal excretion kinetics indicate that it is eliminated by both glomerular filtration and tubular secretion. It is therefore doubtful that high serum and tissue levels of this antibiotic could ever be realistically achieved, and thus the drug would not be useful for deep-seated tissue infections. The renal excretion kinetics, however, suggest that the antibiotic could be useful in treating urinary infection. Nelson states that by performing such pharmacokinetic studies many costly and needless clinical studies would be circumvented.

One other example of the value of pharmacokinetics in toxicological studies is shown by the work of Piper et al. (1973). The herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was administered to rats and dogs in single oral doses of 5 mg/kg with the resultant half-life clearance values being 4.7 and 77.0 h, respectively. It was demonstrated by Piper et al. that these rates of elimination can be correlated with the toxicity of 2,4,5-T in rats and dogs. The slower rate of elimination by dogs than by rats correlated with the higher toxicity in dogs for which the single oral LD_{50} is 100 mg/kg compared to 300 mg/kg for rats (National Institute for Occupational Safety and Health, 1977). These correlations support the statement of Gehring and Young (1978) that there is an inverse relationship between the toxicity of an agent to an individual or species and the ability of the individual or species to eliminate the chemical and a direct relationship between toxicity and the concentrations in the plasma.

In the testing of a chemical for teratogenicity, a knowledge of its pharmacokinetics would be beneficial in explaining the teratogenic effects or lack of such effects. Teratogenic drugs that are dissolved, absorbed, metabolized, and excreted rather quickly and that are administered during the critical period of organogenesis will cause malformations because the action of the drug will occur shortly after administration (Schardein, 1976). An example is β-aminopropionitrile, which is metabolized so quickly to its inactive metabolite, cyanoacetic acid, that if day 15 (the critical period for induction of cleft palate) is not included in the dosing regime, very few malformations are produced in the rat (King, Horigan, and Wilk, 1972). However, there are some teratogenic drugs that are absorbed or metabolized so slowly or incompletely that their teratogenicity would not be manifested if administered during the critical period. An example of this type of action is provided by the antihypercholesterolemic drug triparanol, now withdrawn from use, which has been shown to be an active teratogen in rats when administered as early as day 4 prior to implantation (Roux, 1964, as cited by Schardein, 1976).

2.6.4 Conclusions

The four rate processes of pharmacokinetics are absorption, distribution, metabolism, and elimination. Each of these is important in determining the fate of a chemical in the body and can be influenced by such factors as the physical characteristics of the test substance, pharmacologic properties, and gastrointestinal functions. Knowledge of the pharmacokinetics of a chemical can aid in the hazard assessment process by demonstrating in some cases the lack of potential toxicity because of lack of absorption into the body or a fast elimination from the body and in other cases by explaining why a chemical is more toxic to one species of laboratory animal than to another.

REFERENCES FOR SECTION 2

American Institute of Nutrition. 1977. Ad Hoc Committee Report on Standards for Nutritional Studies. J. Nutr. 107:1340-1348.

Arnold, D. L., S. M. Charbonneau, Z. Z. Zawidzka, and H. C. Grice. 1977. Monitoring Animal Health During Chronic Toxicity Studies. J. Environ. Pathol. Toxicol. 1:227-239.

Boyd, E. M. 1969. Dietary Protein and Pesticide Toxicity in Male Weanling Rats. Bull. WHO 40:801-805.

Braun, W. H., J. C. Ramsey, and P. J. Gehring. 1974. The Lack of Significant Absorption of Methylcellulose, Viscosity 3300 cps from the Gastrointestinal Tract Following Single and Multiple Doses to the Rat. Food Cosmet. Toxicol. 12:373-376.

Brinkman, G. L., and R. F. Miller. 1961. Influence of Cage Type and Dietary Zinc Oxide upon Molybdenum Toxicity. Science 134:1531-1532.

Bryan, G. T., R. R. Brown, J. M. Price. 1964. Incidence of Mouse Bladder Tumors Following Implantation of Paraffin Pellets Containing Certain Tryptophan Metabolites. Cancer Res. 24:582.

Campbell, T., and J. R. Hayes. 1974. Role of Nutrition in the Drug-Metabolizing Enzyme System. Pharmacol. Rev. 26:171-197.

Carroll, K. K., and H. T. Khor. 1970. Effects of Dietary Fat and Dose Level of 7,12-Dimethylbenz(α)anthracene on Mammary Tumor Incidence in Rats. Cancer Res. 30:2260-2264.

Clarke, H. E., M. E. Coates, J. K. Eva, D. J. Ford, C. K. Milner, P. N. O'Donoghue, P. P. Scott, and R. J. Ward. 1977. Dietary Standards for Laboratory Animals: Report of the Laboratory Animals Centre Diets Advisory Committee. Lab. Anim. 11(1):1-28.

Clough, G. 1976. The Immediate Environment of the Laboratory Animal. In: Laboratory Animal Handbooks 7: Control of the Animal House Environment, T. McSheehy, ed. Laboratory Animals, Ltd., Huntingdon, United Kingdom. pp. 77-94.

Coates, M. E., P. N. O'Donoghue, P. R. Payne, and R. J. Ward, eds. 1969. Dietary Standards for Laboratory Rats and Mice. In: Laboratory Animal Handbooks 2. Laboratory Animals, Ltd., London. pp. 1-31.

Cone, M. V., and P. Nettesheim. 1973. Effects of Vitamin A on 3-Methylcholanthrene-Induced Squamous Metaplasias and Early Tumors in the Respiratory Tract of Rats. J. Natl. Cancer Inst. 50:1599-1606.

Dymet, J. 1976. Air Filtration. In: Laboratory Animal Handbooks 7: Control of the Animal House Environment, T. McSheehy, ed. Laboratory Animals, Ltd., Huntingdon, United Kingdom. pp. 209-246.

Edwards, G. S., J. G. Fox, P. Policastro, U. Goff, M. H. Wolf, and D. H. Fine. 1979. Volatile Nitrosamine Contamination of Laboratory Animal Diets. Cancer Res. 39:1857-1858.

Farkas, C. S. 1978. Importance of Interactions Between Nutrients and Environmental Contaminants as a Factor in Experimental Design in Toxicological Research, with Emphasis on Selenium and Ascorbic Acid. Sci. Total Environ. 9(2):148-160.

Fears, T. R., and J. F. Douglas. 1977. Suggested Procedures for Reducing the Pathology Workload in a Carcinogen Bioassay Program, Part I. J. Environ. Pathol. Toxicol. 1:125-137.

Fears, T. R., and J. F. Douglas. 1978. Suggested Procedures for Reducing the Pathology Workload in a Carcinogen Bioassay Program, Part II: Incorporating Blind Pathology Techniques and Analysis for Animals with Tumors. J. Environ. Pathol. Toxicol. 1:211-222.

Festing, M.F.W. 1979. Properties of Inbred Strains and Outbred Stocks, with Special Reference to Toxicity Testing. J. Toxicol. Environ. Health 5:53-68.

Fletcher, J. L. 1976. Influence of Noise on Animals. In: Laboratory Animal Handbooks 7: Control of the Animal House Environment, T. McSheehy, ed. Laboratory Animals, Ltd., Huntingdon, United Kingdom. pp. 51-62.

Flynn, R. J. 1967. The Control of Disease in Laboratory Animals. In: Husbandry of Laboratory Animals, Proceedings of the Third International Committee on Laboratory Animals, M. L. Conalty, ed. Academic Press, New York. pp. 361-372.

Food Safety Council. 1978. Proposed System for Food Safety Assessment. Columbia, Maryland. 136 pp.

Fouts, J. R. 1976. Overview of the Field: Environmental Factors Affecting Chemical or Drug Effects in Animals. Fed. Proc. Fed. Am. Soc. Exp. Biol. 35(5):1162-1165.

Fox, J. G. 1977. Clinical Assessment of Laboratory Rodents on Long Term Bioassay Studies. J. Environ. Pathol. Toxicol. 1:199-226.

Frith, C. H., A. D. Boothe, D. L. Greenman, and J. H. Farmer. 1979. Correlations Between Gross and Microscopic Lesions in Carcinogenic Studies in Mice. J. Environ. Pathol. Toxicol. 3:139-153.

Gehring, P. J., P. G. Watanabe, and G. E. Blau. 1976. Pharmacokinetic Studies in Evaluation of the Toxicological and Environmental Hazard of Chemicals. In: Advances in Modern Toxicology, Vol. 1, Part 1: New Concepts in Safety Evaluation, M. A. Mehlman, R. W. Shapiro, and H. Blumenthal, eds. John Wiley and Sons, New York. pp. 195-270.

Gehring, P. J., and J. D. Young. 1978. Application of Pharmacokinetic Principles in Practice. In: Proceedings of the First International Congress on Toxicology, G. L. Plaa and W.A.M. Duncan, eds. Academic Press, New York. pp. 119-141.

Goldschmidt, S., H. M. Vars, and I. S. Raudin. 1939. Influence of the Foodstuffs upon the Susceptibility of the Liver to Injury by Chloroform, and the Probable Mechanism of Their Action. J. Clin. Invest. 18:277-289.

Grange, P. H. 1976. Design Considerations Relevant to Controlled Environment Buildings. In: Laboratory Animal Handbooks 7: Control of the Animal House Environment, T. McSeehy, ed. Laboratory Animals, Ltd., Huntingdon, United Kingdom. pp. 291-299.

Hatch, A., T. Balazs, G. S. Wiberg, and H. C. Grice. 1965. The Importance of Avoiding Mental Suffering in Laboratory Animals. Animal Welfare Inst. Rep. 14 No. 3.

Heinecke, H. 1967. The Experimental Environment and Its Influence on the Blood Picture of Laboratory Animals. In: Husbandry of Laboratory Animals, Proceedings of the Third International Committee on Laboratory Animals, M. L. Conalty, ed. Academic Press, New York. pp. 447-457.

Hughes, H. C., Jr., and C. M. Lang. 1978. Basic Principles in Selecting Animal Species for Research Projects. Clin. Toxicol. 13(5):611-621.

Hurni, H. 1970. The Provision of Laboratory Animals. In: Methods in Toxicology, G. E. Paget, ed. F. A. Davis Company, Philadelphia. pp. 11-48.

Kalter, H., and J. Warkany. 1959. Experimental Production of Congenital Malformations in Mammals by Metabolic Procedure. Physiol. Rev. 39:69-115.

Kawachi, T., Y. Hirata, and T. Sugimura. 1968. Enhancement of *N*-Nitrosodiethylamine Hepatocarcinogenesis by *L*-Tryptophan in Rats. Gann 59: 523-525.

King, C.T.G., E. Herigan, and A. L. Wilk. 1972. Fetal Outcome from Prolonged Versus Acute Drug Administration in the Pregnant Rat. In: Drugs and Fetal Development, Proceedings of an International Symposium on the Effect of Prolonged Drug Usage on Fetal Development, M. A. Klingberg, A. Abramovici, and J. Chemke, eds. Plenum Press, New York. pp. 61-75.

Knapka, J. J. 1979. Laboratory Animal Feed. Science 204:1367.

Kulwich, B. A., J. F. Hardisty, C. E. Gilmore, and J. M. Ward. 1980. Correlation Between Gross Observations of Tumors and Neoplasms Diagnosed Microscopically in Carcinogenesis Bioassays in Rats. J. Environ. Pathol. Toxicol. 3:281-287.

Lane-Petter, W. 1967. Selection, Training, and Control of Staff. In: Husbandry of Laboratory Animals, Proceedings of the Third International Committee on Laboratory Animals, M. L. Conalty, ed. Academic Press, New York. pp. 61-74.

Madhavan, T. V., and C. Gopalan. 1965. Effect of Dietary Protein on Aflatoxin Liver Injury in Weanling Rats. Arch. Pathol. 80:123-126.

Magee, P. 1970. Tests for Carcinogenic Potential. In: Methods in Toxicology, G. Paget, ed. F. A. Davis Company, Philadelphia. pp. 158-196.

Marshall, W. J., and A.E.M. McLean. 1969. A Requirement for Dietary Lipids for Induction of Cytochrome P-450 by Phenobarbitone in Rat Liver Microsomal Fraction. Biochem. J. 122:569-573.

McLean, A.E.M., and E. K. McLean. 1966. Effect of Diet and 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) on Microsomal Hydroxylating Enzymes and on Sensitivity of Rats to Carbon Tetrachloride Poisoning. Biochem. J. 100:564-571.

McLean, A.E.M., and E. K. McLean. 1969. Diet and Toxicity. Br. Med. Bull. 25:278-281.

McLean, A.E.M., and H. G. Verschuuren. 1969. Effects of Diet and Microsomal Enzyme Induction on the Toxicity of Dimethyl Nitrosamine. Br. J. Exp. Pathol. 50:22-25.

Meister, G., H. P. Hobik, and K.-G. Metzger. 1967. Comparative Bacteriological and Pathological Investigations on Commercially Available SPF-Animals. In: Husbandry of Laboratory Animals, Proceedings of the Third International Committee on Laboratory Animals, M. L. Conalty, ed. Academic Press, New York. pp. 387-391.

Ministry of Health and Welfare Canada. 1975. The Testing of Chemicals for Carcinogenicity, Mutagenicity, and Teratogenicity. Ministry of of Health and Welfare, Ottawa. 183 pp.

Moffitt, A. E., and S. D. Murphy. 1974. Effect of Excess and Deficient Copper Intake on Hepatic Microsomal Metabolism and Toxicity of Foreign Chemicals. In: Trace Substances in Environmental Health, Vol. VII, D. D. Hemphill, ed. University of Missouri Press, Columbia. pp. 205-210.

Mori, K. 1965. Induction of Pulmonary and Uterine Cancers and Leukemia in Mice by Injection of 4-Nitroquinoline-1-oxide. Gann 56:513.

National Academy of Sciences. 1969. Rodents. Standards and Guidelines for the Breeding, Care, and Management of Laboratory Animals. A Report of the Subcommittee on Rodent Standards, Institute of Laboratory Animal Resources. National Academy of Sciences, Washington, D.C. 52 pp.

National Academy of Sciences. 1975. Chemical Carcinogenesis. A Report of the Committee for the Working Conference on Principles for Evaluating Chemicals in the Environment. National Academy of Sciences, Washington, D.C. pp. 134-155.

National Academy of Sciences — National Research Council. 1972. Committee on Animal Nutrition, Subcommittee on Laboratory Animal Nutrition. Nutrient Requirements of Laboratory Animals, 2nd rev. ed. Nutrient Requirements of Domestic Animals, No. 10. Washington, D.C.

National Institute for Occupational Safety and Health. 1977. 2,4,5-Trichlorophenoxyacetic Acid. In: Registry of Toxic Effects of Chemical Substances, Vol. II, E. J. Fairchild, R. J. Lewis, Sr., and R. L. Tatken, eds. Government Printing Office, Washington, D.C. pp. 33-34.

National Research Council. 1977. Chronic Toxicity/Carcinogenicity Tests. In: Principles and Procedures for Evaluation of Toxicity and Household Substances. National Research Council, Washington, D.C. pp. 74-85.

Nelson, R. L. 1976. The Utility of Pharmacokinetics to the Pharmaceutical Industry. J. Clin. Pharmacol. 16:565-569.

Nevins, R. G. 1971. Design Criteria for Ventilation Systems. In: Environmental Requirements for Laboratory Animals. Kansas State University, Manhattan. pp. 28-43.

Newberne, P. M. 1975. Influence on Pharmacological Experiments of Chemicals and Other Factors in Diets of Laboratory Animals. Fed. Proc. 34(2):209-218.

Newberne, P. M., and A. E. Rogers. 1976. Nutritional Modulation of Carcinogenesis. In: Fundamentals in Cancer Prevention, P. N. Magee et al., eds. University of Tokyo Press, Tokyo/University Park Press, Baltimore. pp. 15-40.

Newberne, P. M., A. E. Rogers, and G. N. Wogan. 1968. Hepatorenal Lesions in Rats Fed a Low Lipotrope Diet and Exposed to Aflatoxin. J. Nutr. 94:331-343.

Page, N. D. 1977. Concepts of a Bioassay Program in Environmental Carcinogenesis. In: Advances in Modern Toxicology, Vol. 3: Environmental Cancer, H. F. Kraybill and M. A. Mehlman, eds. John Wiley and Sons, New York. pp. 87-171.

Piper, W. N., J. Q. Rose, M. L. Leng, and P. J. Gehring. 1973. The Fate of 2,4,5-Trichlorophenoxyacetic Acid (2.4.5-T) Following Oral Administration to Rats and Dogs. Toxicol. Appl. Pharmacol. 26:339-351.

Porter, G. 1967. Assessment of Environmental Influence on the Biological Responses of the Animal. In: Husbandry of Laboratory Animals, Proceedings of the Third International Committee on Laboratory Animals, M. L. Conalty, ed. Academic Press, New York. pp. 29-42.

Porter, G., W. Lane-Petter, and N. Horne. 1963. Assessment of Diets for Mice. Z. Versuchstierkd. 2:75-91.

Pott, F., A. Brockhaus, and F. Huth. 1973. Tests on the Production of Tumors in Animal Experiments with Polycyclic Aromatic Hydrocarbons. Zbl. Bakt. Hyg., I. Abt. Orig. B 157:34-43.

Prieur, D. J., D. M. Young, R. D. Davis, D. A. Cooney, E. R. Homan, R. L. Dixon, and A. M. Guarino. 1973. Procedures for Preclinical Toxicologic Evaluation of Cancer Chemotherapeutic Agents: Protocols of the Laboratory of Toxicology. Cancer Chemother. Rep. Part 3 4(1):1-30.

Roe, F.J.C. 1965. Spontaneous Tumors in Rats and Mice. Food Cosmet. Toxicol. 3:717-720.

Rogers, A. E. 1975. Variable Effects of a Lipotrope-Deficient High-Fat Diet on Chemical Carcinogenesis in Rats. Cancer Res. 35:2469-2474.

Rogers, A. E., O. Sanchez, F. M. Feinsod, and P. M. Newberne. 1974. Dietary Enhancement of Nitrosamine Carcinogenesis. Cancer Res. 34:96-99.

Saffiotti, U., R. Montesano, A. R. Sellakumar, and S. A. Borg. 1967. Experimental Cancer of the Lung: Inhibition by Vitamin A of the Induction of Tracheobronchial Squamous Metaplasia and Squamous Cell Tumors. Cancer 20:857-864.

Schardein, J. L. 1976. Drugs as Teratogens. The Chemical Rubber Company, Cleveland, Ohio. 291 pp.

Shaw, B. H. 1976. Air Movements Within Animal Houses. In: Laboratory Animal Handbooks 7: Control of the Animal House Environment, T. McSheehy, ed. Laboratory Animals, Ltd., Huntingdon, United Kingdom. pp. 185-208.

Short, D. J. 1967. Handling, Sexing and Palpating Laboratory Animals. In: Husbandry of Laboratory Animals, Proceedings of the Third International Symposium of the International Committee on Laboratory Animals, M. L. Conalty, ed. Academic Press, New York. pp. 3-15.

Silverstone, H., and A. Tannenbaum. 1951. Proportion of Dietary Protein and the Formation of Spontaneous Hepatomas in the Mouse. Cancer Res. 11:442-446.

Smith, D. M., A. E. Rogers, B. J. Herndon, and P. M. Newberne. 1975. Vitamin A (Retinyl Acetate) and Benzo(*a*)pyrene-Induced Respiratory Tract Carcinogenesis in Hamsters Fed a Commercial Diet. Cancer Res. 35:11-16.

Smith, D. M., A. E. Rogers, and P. M. Newberne. 1975. Vitamin A and Benzo(*a*)pyrene Carcinogenesis in the Respiratory Tract of Hamsters Fed a Semisynthetic Diet. Cancer Res. 35:1485-1488.

Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U.S. Department of Health, Education, and Welfare Publ. No. (NIH)76-801. 65 pp.

Surthin, A., and K. Yagi. 1958. Distribution in Renal Cell Fractions of Sulfhydryl Groups in Rats on Normal and Sucrose Diets and Its Relation to Renal Mercury Distribution After Mercuric Chloride Injection. Am. J. Physiol. 192:405-409.

Swann, P. F., and A.E.M. McLean. 1968. The Effect of Diet on the Toxic and Carcinogenic Action of Dimethylnitrosamine. Biochem. J. 107:14-15.

U.S. Department of Health, Education, and Welfare. 1974. Guide for the Care and Use of Laboratory Animals, 4th ed. National Institutes of Health. DHEW Publ. No. (NIH)74-23, Washington, D.C. 56 pp.

U.S. Food and Drug Administration. 1971. Panel on Carcinogenesis Report on Cancer Testing in the Safety Evaluation of Food Additives and Pesticides. Toxicol. Appl. Pharmacol. 20:419-438.

van der Waaij, D., and D. W. van Bekkum. 1967. Resident Infection in Laboratory Animal Colonies and Their Influence on Experiments. In: Husbandry of Laboratory Animals, Proceedings of the Third International Committee on Laboratory Animals, M. L. Conalty, ed. Academic Press, New York. pp. 373-386.

Walker, E. A., M. Castegnaro, and L. Griciute. 1979. *N*-nitrosamines in the Diet of Experimental Animals. Cancer Lett. 6(3):175-178.

Weihe, W. H. 1976*a*. The Effects on Animals of Changes in Ambient Temperature and Humidity. In: Laboratory Animal Handbooks 7: Control of the Animal House Environment, T. McSheehy, ed. Laboratory Animals, Ltd., Huntingdon, United Kingdom. pp. 41-50.

Weihe, W. H. 1976*b*. Influence of Light on Animals. In: Laboratory Animal Handbook 7: Control of the Animal House Environment, T. McSheehy, ed. Laboratory Animals, Ltd., Huntingdon, United Kingdom. pp. 63-76.

Weisburger, J. H. 1976. Bioassays and Tests for Chemical Carcinogens. In: Chemical Carcinogens, C. E. Searle, ed. American Chemical Society Monograph 173. pp. 1-23.

Winter, C. A., and L. Flataker. 1962. Cage Design as a Factor Influencing Acute Toxicity of Respiratory Depressant Drugs in Rats. Toxicol. Appl. Pharmacol. 4:650-655.

Withey, J. R. 1978. Pharmacokinetic Principles. In: Proceedings of the First International Congress on Toxicology, G. L. Plaa and W.A.M. Duncan, eds. Academic Press, New York. pp. 97-118.

World Health Organization. 1978. Principles and Methods for Evaluating the Toxicity of Chemicals, Part I: Environmental Health Criteria 6. Geneva. 273 pp.

Young, J. F., and J. F. Holson. 1978. Utility of Pharmacokinetics in Designing Toxicological Protocols and Improving Interspecies Extrapolation. J. Environ. Pathol. Toxicol. 2:169-186.

Zbinden, G. 1963. Experimental and Clinical Aspects of Drug Toxicity. Adv. Pharmacol. 2:1-112.

Zbinden, G. 1976. The Role of Pathology in Toxicity Testing. In: Progress in Toxicology, Special Topics, Vol. 2. Springer-Verlag, New York. pp. 8-18.

3. ACUTE TOXICITY

3.1 INTRODUCTION

In toxicology the term acute is defined as a single dose or exposure, or fractions of a dose, administered over a short period (National Academy of Sciences, 1977). The toxic effects of such a dosing regime may be observed within a few minutes or up to several days after dosing. The effects may vary greatly with sex and age of the animal as well as within and between different strains and species.

In acute oral LD_{50} studies the test substance is commonly given by gavage in a single dose or divided doses within 1 day, and the animals are observed for 2 weeks; however, when possible, the route of exposure should be that which is likely to be encountered in humans. The number of deaths, time of death, clinical signs, and necropsy are parameters used in assessing the acute toxicity of the chemical.

The Food Safety Council (1978) lists the following three purposes for conducting acute toxicity tests of chemicals:

1. to give a quantitative measure of acute toxicity (LD_{50}) for comparison with other substances,
2. to identify the clinical manifestations of acute toxicity, and
3. to give dose-ranging guidance for other tests.

This section will discuss aspects of acute toxicity such as the LD_{50}, necropsy as a part of the protocol of an acute toxicity test, and the length of the observation period following acute administration of the test chemical.

3.2 LD_{50} DETERMINATIONS

3.2.1 Introduction

The most frequently determined index of toxicity is the LD_{50}, the dose that is lethal to one-half of a group of treated animals (National Academy of Sciences, 1977). In addition, the LD_{50} can be used as a basis for determining doses to be used in subsequent subchronic and chronic toxicity studies. It can also aid the researcher in determining which species and route of administration would be the most suitable for his or her experiments. The following sections will show how the choice of species, route of administration, and sex of the animal may affect the LD_{50} and acute toxicity response.

The information presented on acute LD_{50}s is available in the National Institute for Occupational Safety and Health (NIOSH) *Registry of Toxic Substances* (1977), which has entries in the following format:

BRO9500 Ammonium, (2-hydroxyethyl)diisopropylmethyl-, bromide, xanthene-9-carboxylate

TXDS: Oral-rat LD_{50}: 370 mg/kg
Intraperitoneal-rat LD_{50}: 25 mg/kg
Subcutaneous-rat LD_{50}: 298 mg/kg
Intraduodenal-rat LD_{50}: 125 mg/kg
Oral-mouse LD_{50}: 445 mg/kg
Intraperitoneal-mouse LD_{50}: 78 mg/kg
Oral-rabbit LD_{50}: 750 mg/kg
Intravenous-guinea pig LD_{Lo}: 51 mg/kg

This format provides information on the route of administration, the species, and the toxic LD_{50} dose or LD_{Lo} dose (lowest published lethal dose reported). Also included in the NIOSH document are synonyms, Chemical Abstracts Service registry numbers, and the reference for each item of toxicity data. Table 3.1 is a list of 96 compounds, arbitrarily chosen, for which the LD_{50} values were examined and compared for species differences and for differences between routes of administration. Although not discussed, it is recognized that the differences in reported LD_{50} values may have also been influenced by the age and strain of the test animals, duration of the starvation period prior to dosing, ambient temperature, concentration of the test substance, speed of injection, and type of solvent or suspension medium (Zbinden, 1973), as well as by interlaboratory variations in experimental procedures.

Table 3.1. Compounds examined for LD$_{50}$ differences between species and between routes of administration

Ammonium (2-hydroxyethyl)diisopropylmethyl-, bromide, xanthene-9-carboxylate
Ammonium [(p-hydroxy-2-isobutyl)phenyl]trimethyl-, iodide, methylcarbamate
Arsine, dichloro(2-chlorovinyl)-
Benzaldehyde, p-isopropyl-
Benzamide, 4-amino-N-cyclopropyl-3,5-dichloro-
Benzimidazole, 2-(4-thiazolyl)-, hydrochloride
2-Benzimidazolinone, 5-chloro-1-[3-(dimethylamino)propyl]-3-phenyl-
Benzoic acid, benzyl ester
Benzoic acid, 2,3,6-trichloro-
2-H-Benzo(a)quinolizin-2-one, 1,3,4,6,7,11b-hexahydro-3-isobutyl-9,10-dimethoxy-
Benzoxazole, 2-amino-5-chloro-
Benzoxazoline, 3-methyl-2-(methylamino)-
2-Benzoxazolinone, 5-chloro-
Benzyl alcohol, alpha-(1-aminoethyl)-, hydrochloride, (+ -)-
Carbamic acid, methyl-, m-tolyl ester
Carbamic acid, methyl-, 3,4,5-trimethylphenyl ester
Carbanilic acid, m-chloro-, 4-chloro-2-butynyl ester
Carbon tetrachloride
2-Carboxymethylmercaptobenzenestibonic acid
Cerium chloride
Ethylamine, N-methyl-2-[(o-methyl-alpha-phenylbenzyl)oxy]-,hydrochloride
Ethylene glycol
Guanidine, 1-cyano-3-tert-pentyl-
3-Heptanone, 6-(dimethylamino)-4,4-diphenyl-, hydrochloride
1,6-Hexanediamine, N,N,N',N'-tetramethyl-, polymer with 1,3-dibromopropane
Hydrazine, methyl-, hydrochloride
Hydroquinone
Imidazole
3-3-Imidazoline, 4-amino-2,2,5,5-tetrakis(trifluoromethyl)-
1,3-Indandione, 2-[(p-chlorophenyl)phenylacetyl]-
1H-Indazole, 1-benzyl-3-[3-(dimethylamino)propoxy]-, monohydrochloride
Indene-1-ethylamine, N,N-dimethyl-1-phenyl-, hydrochloride
Indole-3-acetic acid, 1-(p-chlorobenzoyl)-5-methoxy-2-methyl-
Indole, 3-(2-aminoethyl)-5-methoxy-, hydrochloride
Isonicotinic acid, 2-[2-(benzylcarbamoyl)ethyl]hydrazide
Isonicotinic acid, 2-isopropylhydrazide
Isonipecotamide, 4-(p-chlorophenyl)-1-[3-(p-fluorobenzoyl)propyl]-N,N-dimethyl-
Isonipecotic acid, 1-(p-aminophenethyl)-4-phenyl-, ethyl ester
Meglumine hydroxamate
Mercury, [3-(alpha-carboxy-o-anisamide)-2-(2-hydroxyethoxy)propyl]hydroxy-, monosodium salt
Mercury, (3-cyanoguanidino)methyl-
Mercury (II) iodide
4-Metathiazanone, 2-(3,4-dichlorophenyl)-3-methyl-, 1, 1-dioxide
Methanesulfoanilide, 4'-[1-hydroxy-2-(methylamino)propyl]-, hydrochloride
Methanesulfonic acid, iodo-, sodium salt
2,6-Methano-3-benzazocin-8-ol, 1,2,3,4,5,6-hexahydro-3-allyl-6-ethyl-11-methyl-
4,7-Methanoindene, 1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-

Table 3.1 (continued)

Monomethylhydrazine nitrate
Morphinan-3, 14-diol, 17-(cyclopropylmethyl)-
Morphinan-3,6-alpha-diol, 7,8-didehydro-4,5-alpha-epoxy-17-methyl-
Morphinan, 17-(p-nitrophenethyl)-, hydrochloride (+ -)-
Morphinan-6-alpha-ol, 7,8-didehydro-4,5-alpha-epoxy-3-methoxy-17-methyl-
2-Naphthalenemethanol, alpha-[(isopropylamino)methyl]-
Naphthalimide, N-hydroxy-, diethyl phosphate
2-Naphthol, 1,2,3,4-tetrahydro
Neomycin
Nickel (II) acetate(1:2)
Nicotinamide, N,N-diethyl-
2-Norbornanamine, N-ethyl-3-phenyl-, hydrochloride
2-Norbornanamine, N-3,3-trimethyl-
2-Norbornanamine, N,2,3,3-tetramethyl-, hydrochloride
1-Oxa-3,9-diazaspiro(5.5)undecan-2-one, 3-ethyl-9-[3-(p-fluorobenzoyl)propyl]-
1,2,4-Oxadiazole, 5-[2-(diethylamino)ethyl]-3-phenyl-
1,2,4-Oxadiazolidine-3,5-dione, 2-(3,4-dichlorophenyl)-4-methyl-
2H-1,3-Oxazine-2,4(3H)-dione, 5,5-diethyldihydro-
Pactomycin
Penicillanic acid, 6-phenoxyacetamido-
2,4-Pentanediol, 2-(p-chlorophenyl)-4-methyl-
4-Pentenoic acid, 2-[(2-diethylamino)ethyl]-2-phenyl-, ethyl ester
1-Penten-4-yn-3-ol, 1-chloro-3-ethyl
Phenazine, 3-amino-7-(dimethylamino)-2-methyl-, hydrochloride
Phenethyl alcohol
Phenol, 2,4-dinitro-
Phenol, 2,4,5-trichloro-
Phenothiazine, 2-chloro-10-[3-(5-cyclopentyl-5-methyl)aminopropyl]-
Phenothiazine, 10-[2-(diethylamino)propyl]-
Phosphonothioic acid, phenyl-, O-ethyl O-(p-nitrophenyl) ester
Phosphoramidothioic acid, O,S-dimethyl ester
Phosphoric acid, 2-chloro-1-(2,4-dichlorophenyl)vinyl diethyl ester
1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-
12,-Propanediol, 3-(o-methoxyphenoxy)-, 1-carbamate
Pyrrolidinium, 3-hydroxy-1,-dimethyl-, bromide,alpha-cyclopentylamandelate
3-Pyrrolidinol, 1,2-dimethyl-3-phenyl-, propionate
Quinine, hexylbromide
Sodium pentafluorostannite
Streptomycin
Succinic acid, cadmium salt (1:1)
Sulfanilamde
Sulfanilamide, N^1-2-thiazolyl-
5H-Tetrazoloazepine, 6,7,8,9-tetrahydro-
Theophylline, 8-benzyl-7-[2-(ethyl(2-hydroxyethyl)amino)ethyl]-,hydrochloride
2H-1,3,5-Thiadiazine-2-thione, tetrahydro-3,5-dimethyl-
s-Triazine, 2-azido-4-(isopropylamino-6-(methylthio)-
s-Triazine,2,4-dichloro-6-(o-chloroanilino)-
Urea, 3-(hexahydro-4,7-methanoindan-5-yl)-1,1-dimethyl-
Urea, 1,1,3,3-tetramethyl-

3.2.2 Difference Between Species

Table 3.2 uses the LD_{50} data for each compound listed in Table 3.1 to show the LD_{50} differences (expressed as percentage) between different species categorized by route of administration. For example, the first entry in Table 3.2, cat, is compared with the pigeon and rat for the oral route of administration. The comparison of the cat with the rat indicates that for one compound the cat had an LD_{50} greater than the rat LD_{50} by 1% to 25%, for three compounds the rat LD_{50} was greater than the cat LD_{50} by 51% to 75%, and for one other compound the rat LD_{50} was greater by 76% to 100%. Therefore, for four of the five chemicals compared, the rat showed a higher tolerance to the acute dose. Collective examination of data in Table 3.2 reveals that 5.8% of the species compared showed no difference in LD_{50} amounts, 23.3% of the species had LD_{50} values differing by 1% to 25%, 29.4% had differing LD_{50} values of 26% to 50%, 27.7% of the species had LD_{50} values that differed by 51% to 75%, and 13.9% of the species had LD_{50} differences between 76% and 100%. Therefore, less than one-half of the 374 species comparisons (41.7%) showed LD_{50} values that differed by more than 50%. A more detailed examination of the data in Table 3.2 reveals the following:

1. The rat and mouse appear to be less sensitive indicators of toxicity than the dog.
2. The rabbit is a less sensitive toxicity indicator than the rat but slightly more sensitive than the mouse.
3. The mouse is a less sensitive toxicity indicator than the hamster.
4. The differences between LD_{50} values for the mouse and rat were less than 50% for 56% of the comparisons.
5. The mouse has a larger LD_{50} (less sensitivity) than the rat in the majority of comparisons (60%).

The principal observations from these data are that the mouse and the rat represent the most commonly used test species (84.3% of all comparisons involved one or both) and that for slightly more than one-half (58%) of all the species comparisons the LD_{50} values differed by less than 50%.

Table 3.2. Percent (%) differences between species

Species	Route of administration	Percent differences 0	 1 to 25	 26 to 50	 51 to 75	 76 to 100
Cat						
Pigeon	Oral					Pigeon[1]
Rat	Oral		Cat		Rat(3)[2]	Rat
Chicken						
Guinea pig	Oral		Guinea pig	Guinea pig		
Hamster	Oral			Hamster		
Mouse	Subcutaneous		Mouse		Mouse	
	Oral		Mouse(2)[2]		Mouse	
Pigeon	Oral				Pigeon	
	Intravenous				Pigeon	
Quail	Oral			Quail		
Rabbit	Oral					Rabbit
Dog						
Cat	Oral		Cat		Dog	
Guinea pig	Subcutaneous			Dog(2)		
	Oral				Guinea pig	Dog
Hamster	Oral			Dog		
Monkey	Subcutaneous			Monkey		
	Oral					Monkey
Pigeon	Oral			Pigeon		
Rabbit	Subcutaneous	2[3]	Dog			
	Skin				Dog	
	Intravenous			Dog	Dog	
	Oral	1[2]		Rabbit		
	Intraperitoneal				Rabbit	
Rat	Oral			Rat, dog(2)[2]	Rat, dog(2)[2]	Rat, dog
	Subcutaneous		Rat	Dog		
	Intravenous		Rat	Rat	Rat, dog	Dog
	Intraperitoneal	1[2]		Rat(2)[2]		
	Skin			Rat		
Duck						
Chicken	Intravenous		Chicken		Chicken	
Dog	Oral					Dog
Mouse	Intravenous					Duck
Pigeon	Oral		Pigeon			Pigeon
	Intravenous					Pigeon
Quail	Oral			Quail	Quail	

Table 3.2 (continued)

Species	Route of administration	Percent differences 0	1 to 25	26 to 50	51 to 75	76 to 100
Guinea pig						
Cat	Oral				Cat, guinea pig	Guinea pig
Hamster	Intraperitoneal	1				
	Oral		Guinea pig			
Monkey	Subcutaneous				Monkey	
Mouse	Oral	1	Mouse([illegible])	Mouse([illegible]), guinea pig([illegible])	Guinea pig	Mouse(2)
	Intraperitoneal			Mouse, guinea pig	Guinea pig	
	Subcutaneous		Guinea pig	Guinea pig([illegible])		
	Intravenous	1				
Pigeon	Oral			Guinea pig		
Rabbit	Subcutaneous			Rabbit		
	Skin			Guinea pig		Guinea pig
	Oral		Guinea pig	Rabbit, guinea pig	Rabbit(2), guinea pig	
Rat	Oral	1	Guinea pig([illegible]), rat	Guinea pig([illegible]), rat([illegible])	Guinea pig(4), rat([illegible])	Rat
	Subcutaneous	1	Rat		Guinea pig, rat	Guinea pig
	Skin			Guinea pig		
	Intraperitoneal		Guinea pig			
	Intravenous				Rat	
Mouse						
Cat	Oral			Cat	Mouse([illegible])	Mouse
	Intraperitoneal			Mouse		
Dog	Oral		Dog, mouse	Mouse([illegible])	Mouse	Mouse([illegible])
	Subcutaneous				Dog	
	Intraperitoneal			Mouse	Mouse	
	Intravenous		Mouse	Mouse	Mouse	Mouse
Hamster	Oral		Mouse([illegible])			
	Intraperitoneal		Mouse	Mouse	Hamster	
Monkey	Subcutaneous				Monkey	
	Oral		Mouse			
Pigeon	Intravenous		Mouse			
Rabbit	Oral		Rabbit([illegible])	Rabbit([illegible]), mouse([illegible])	Mouse([illegible]), rabbit([illegible])	Rabbit([illegible])
	Subcutaneous				Mouse([illegible])	
	Intravenous			Mouse, rabbit([illegible])	Mouse([illegible])	Mouse([illegible])
Quail						
Dog	Oral				Dog	
Pigeon	Oral		Pigeon		Pigeon	
Rat	Oral			Rat	Rat	

Table 3.2 (continued)

Species	Route of administration	Percent differences 0	1 to 25	26 to 50	51 to 75	76 to 100
Rabbit						
Cat	Oral		Cat			
Duck	Oral			Duck		
Monkey	Subcutaneous			Monkey		
	Oral					Monkey
Rat						
Duck	Oral				Rat	Rat, duck
	Intravenous		Duck			
Hamster	Oral		Rat, hamster	Hamster, rat		
Rat	Intraperitoneal		Hamster(1)		Hamster(1)	
	Skin		Hamster			
Monkey	Subcutaneous			Monkey		
	Oral			Monkey		
Mouse	Oral	4	Mouse(9), rat(8)	Mouse(3), rat(6)	Mouse(7), rat(10)	Mouse(1), rat(1)
	Intraperitoneal	1	Mouse(1), rat(4)	Mouse(4), rat(1)	Mouse(1), rat(2)	Mouse
	Intravenous	1	Mouse(5), rat(2)	Mouse(3), rat(4)	Mouse(3), rat	Mouse
	Subcutaneous		Mouse(3)	Mouse(2), rat(2)	Mouse(2), rat(3)	Mouse
	Intramuscular				Mouse	
Pigeon	Oral		Rat(3)			
	Intravenous					Pigeon
Rabbit	Oral	2	Rabbit(5)	Rabbit	Rat(11), rat	Rabbit(3), rat
	Subcutaneous			Rat, rabbit		
	Intraperitoneal	1		Rat, rabbit		
	Intravenous	2	Rat, rabbit	Rat(2), rabbit		
	Skin		Rabbit		Rat, rabbit	Rat, rabbit(2)
	Intramuscular			Rat		

[a] Indicates which animal of the comparison had the greatest LD_{50}. Position of species indicates the percent differences in the LD_{50}s of the two species being compared.

[b] Represents number of chemicals compared where LD_{50} of species indicated was greater than species being compared. If there is no number then only one chemical was compared.

[c] The number indicates how many times the LD_{50} values were identical for the species compared.

3.2.3 Difference Between Administration Routes

Table 3.3 shows the percentage difference between LD_{50} values for various pairs of routes of administration categorized by species. Abbreviations in the appropriate columns indicate which route of the two being compared is the greater. For example, the "inp" (intraperitoneal) notation in the first entry for the mouse at 76% to 100% indicates that the LD_{50} for the intraperitoneal route of administration was 76% to 100% greater than the LD_{50} for the intracerebral route for the same compound. Column by column examination of the data in Table 3.3 reveals that 2.4% of the entries fall in the 0% (no difference in LD_{50}) column, 20.4% in the 1% to 25% column, 20.4% in the 26% to 50% column, 26.2% in the 51% to 75% column, and 29.8% in the 76% to 100% column. These data indicate that the LD_{50} can vary significantly depending on the administration route.

A more detailed examination of the data in Table 3.3 shows the following:

1. For all compounds, the subcutaneous route of administration was always associated with a higher LD_{50} than the intravenous route.
2. Of the 328 entries, 116 (35%) compared the subcutaneous route with other routes, 140 (42.6%) compared intravenous administration with other routes, and 199 (60.6%) compared the oral route of administration with other routes. Thus the predominant use of the oral administration route is shown.
3. When the intravenous route was compared to the oral and the intraperitoneal routes, respectively, the LD_{50} values were greater for the oral and intraperitoneal routes for most of the compounds. In fact, in only four of 56 comparisons (two being equal) was the intravenous LD_{50} greater than the oral LD_{50} [those four compounds were methylhydrazine hydrochloride, 2-isopropylhydrazide isonicotinic acid, pactamycin, and 2-chloro-1-(2,4-dichlorophenyl)vinyl phosphoric acid diethyl ester], and in only five of 35 comparisons was the intravenous LD_{50} greater than the intraperitoneal route [those four compounds (two comparisons involved the same compound) were methylhydrazine hydrochloride, 4-amino-2,2,5,5-tetrakis

Table 3.3. Percent LD_{50} differences between routes of administration

Routes of administration	Species	Percent differences				
		0	1 to 25	26 to 50	51 to 75	76 to 100
Intracerebral and intraperitoneal	Mouse					Inp
Intracerebral and intravenous	Mouse					Inv
Intracerebral and subcutaneous	Mouse					Sub
Intraperitoneal and intramuscular	Mouse		Inm, Inp[a]			
	Rat			Inm		
Intraperitoneal and skin	Rat				Skin	Skin(2)[b]
	Hamster					Skin
	Guinea pig					Skin
Intraperitoneal and subcutaneous	Rat	1[c]		Inp, Sub	Sub(3)[b]	Sub(2)[b]
	Mouse		Sub(5),[b] Inp(3)[b]	Sub(7)[b]	Sub(3)[b]	Sub
Intravenous and intramuscular	Rabbit					Inm
	Rat		Inm			Inm
	Mouse		Inv, Inm			
Intravenous and intraperitoneal	Rat		Inv, Inp	Inp(4)[b]	Inp(4)[b]	Inp(2)
	Mouse		Inv(3),[b] Inp(3)[b]	Inp(7)[b]	Inp(8),[b] Inv	Inp
Intravenous and oral	Rat		Inv	0[e]	0(5)[b]	0(11)[b]
	Mouse	1[c]	0(4),[b] Inv	0(2)[b]	0(7),[b] Inv(2)[b]	0(18)[b]
	Dog			0		
	Guinea pig					0
	Rabbit	1[c]				
Intravenous and skin	Rabbit					Skin
	Rat					Skin
Intravenous and subcutaneous	Rabbit				Sub	Sub(2)[b]
	Mouse		Sub(2)[b]	Sub(6)[b]	Sub(8)[b]	Sub(7)[b]
	Rat		Sub(2)[b]	Sub	Sub(3)	Sub(7)[b]
	Guinea pig					Sub

Table 3.3 (continued)

Routes of administration	Species	Percent differences				
		0	1 to 25	26 to 50	51 to 75	76 to 100
Oral and intracerebral	Mouse					0
Oral and intramuscular	Rat			Inm		0
	Mouse	1[c]		0		
Oral and intraperitoneal	Rat	2[c]	0(5),[b] Inp(2)[b]	0(6),[b] Inp	0(10)[b]	0(12),[b] Inp
	Mouse		0(7)[b]	0(7),[b] Inp	0(11),[b] Inp(2)[b]	0(9)[b]
	Hamster			0	0(3)[b]	
	Cat		Inp			
	Rabbit		Inp			
	Guinea pig	1[c]				
Oral and subcutaneous	Rat		0(9)[b]	0(3),[b] Sub	Sub	0
	Mouse		0(6),[b] Sub(2)[b]	0(7),[b] Sub	0(4),[b] Sub(3)[b]	
	Guinea pig	1[c]				
Skin and oral	Rat			Skin	0, Skin(3)[b]	Skin(5)[b]
	Mouse					0
	Rabbit		0		Skin	
	Hamster					Skin
	Guinea pig					Skin
Subcutaneous and intramuscular	Rabbit		Inm			
	Mouse		Sub	Sub		
Subcutaneous and skin	Rat			Skin		Skin
	Dog					Skin
	Rabbit				Skin	
	Guinea pig					Skin

[a] Inm – intramuscular; Inp – intraperitoneal; Inv – intravenous; 0 – oral; Sub – subcutaneous.

[b] Represents number of comparisons when route indicated was greater than the route being compared. If no number is present then only one chemical was compared.

[c] The number indicates how many times the LD_{50} values were identical in a comparison.

(trifluoromethyl)-3-imidazoline, 2-isopropylhydrazide isonicotinic acid, and N^1-2-thiazolylsulfanilamide].

4. The oral LD_{50} was greater than the subcutaneous LD_{50} for 82.5% of the comparisons; however, the difference was generally less than 50%.
5. A comparison between the oral LD_{50} and intraperitoneal LD_{50} showed that for 89% of the entries the oral route had a greater LD_{50} than the intraperitoneal route [those compounds for which the intraperitoneal route was greater for at least one species were 2,3,6-trichlorobenzoic acid, alpha-(1-aminoethyl)benzyl alcohol hydrochloride, 1-cyano-3-tert-pentylguanidine, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid, pactamycin, tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione, 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine, 4-amino-*N*-cyclopropyl-3,5-dichlorobenzamide, and 2-(3,4-dichlorophenyl)-3-methyl-4-metathiazanone-1,1-dioxide].

The principal conclusion from these data is that the choice of route of administration is an important part of the experimental design because it can result in significant differences in toxicity response. This emphasizes the importance of selecting the route of exposure that will be the likely exposure route in man when conducting safety assessments of chemicals.

3.2.4 Sex Differences in the Laboratory Rat

Male and female rats show differing susceptibilities to the acute doses of some chemicals. Hayes (1975) states that the rat, which is used more than any other species for studies in toxicology, apparently shows more variation between the sexes in its response to chemicals than does any other species. One example is provided by the research of Kast et al. (1975*b*) with the amino-halogen-substituted benzylamine, fominoben-HCl. The oral LD_{50} for males was 6450 mg/kg and for females, 4500 mg/kg. Other examples of sex differences in LD_{50} response are seen in the research of Gaines (1960, 1969). He reports that, in acute oral toxicity tests with 98 pesticides, most compounds were more toxic to female than to male rats. This is demonstrated with the organophosphorus pesticides azodrin (male LD_{50}, 126 mg/kg and female LD_{50}, 112 mg/kg), Bayer 37289 (male LD_{50}, 180 mg/kg and female LD_{50}, 64 mg/kg), and ethion (male LD_{50}, 245 mg/kg and female LD_{50}, 62 mg/kg). Hayes (1975) used the oral toxicity LD_{50} data of Gaines (1960, 1969) on the rat for 69 pesticides and calculated the ratio of female to male oral LD_{50}s. His calculations showed that the ratio ranged from 0.21 (indicating greater susceptibility of the female) to 4.62 (indicating greater susceptibility of the male) and averaged 0.94. Therefore, these studies indicate that both males and females should be used as test animals when the rat is the animal of choice for the acute toxicity testing of a chemical.

3.2.5 Test-Limiting Criteria

Some chemicals are relatively innocuous when given as single doses. If this relative lack of acute toxicity can be determined at the beginning of the acute toxicity test, there is no need for continuing the acute testing. The National Academy of Sciences (1977) states that, for most purposes, if animals survive single oral doses of 5 or 10 g/kg, an adequate estimate of hazard is obtained. The Interagency Regulatory Liaison Group's guidelines indicate that no further acute oral toxicity testing of a chemical is necessary if no mortality is seen at a dose of 5 g/kg. Similarly, the guidelines for the Organization for Economic Cooperation and Development state that, if 5 of 10 animals survive a dose of 5 g/kg of a chemical for 14 days, no further acute toxicity testing is necessary. If these criteria are met in one sex but not the other acute toxicity testing must proceed with the sex that did not meet the criteria for limiting the acute test.

3.2.6 Conclusions

The information in Sect. 3.2.2 and Sect. 3.2.3 clearly indicates the importance of the selection of proper species and route of administration. Specifically, 42% of all species comparisons (Table 3.2) indicated differences in LD_{50} values of more than 50%, and 56% of all comparisons involving administration routes (Table 3.3) showed differences between LD_{50} values of more than 50%. When the laboratory rat is the test animal in acute toxicity studies, both male and female animals should be used because of the variation in response between the sexes. If a chemical does not cause more than 50% mortality when administered in acute doses of 5 g/kg, further acute toxicity testing is not required. For safety assessment of chemicals with potential human exposure, it is desirable to select a test animal that will reflect what is anticipated to occur in man. In practice, however, the rat or mouse is usually the species of first choice because they are easy to work with and relatively inexpensive and there is a vast amount of information available on which to base comparative assessments. The difference in LD_{50} values as a result of using different exposure routes indicates the necessity of selecting the exposure route that will be the likely exposure route in man when chemicals are tested that have a potential for human exposure.

3.3 HUMAN VS ANIMAL RESPONSE

3.3.1 Introduction

This section will compare the responses of animals and humans to acute exposures of chemical substances. For the most part, information on dermal and ocular toxicity will not be presented, as this will be covered in detail in a companion document.

3.3.2 Comparison of Lowest Published Lethal Doses (LD_{Lo})

The source of the information in this section is the *NIOSH Registry of Toxic Substances* (1977) in which the LD_{Lo} is defined as the lowest published lethal dose. Twenty-seven compounds (Table 3.4) were chosen at random and the human and animal LD_{Lo} data compared in Table 3.5. The following observations are evident:

1. With the exception of two compounds, all comparisons involved the oral route of administration.
2. In only one instance was the human and animal LD_{Lo} equal, whereas 8.6% of the comparisons had LD_{Lo} differences between 1% and 25%, 17.3% between 26% and 50%, 15.2% between 51% and 75%, and 60.8% between 76% and 100%.

Table 3.4. Compounds selected for human vs animal LD_{Lo} comparisons

Chemical[a]	Dose (mg/kg)
Oxalic acid	0.071
Quinine, monohydrochloride	0.230
Hydrocyanic acid	0.570
Phenol, 2,4-dinitro	4.3
Barbituric acid, 5-ethyl-5-hexyl-, sodium salt	5
Ouabain	5
Phosphoramidocyanidic acid, dimethyl-, ethyl ester	23
Mercury (11) chloride	29
Phenol, pentachloro-	29
Strychinine	30
Carbon tetrachloride	43
Chloral hydrate	50
Veriloid	50
Zinc, bis(dimethyldithiocarbamato)-	50
Quinine	50
Benzamide, *p*-amino-*N*-(2-diethylamino)ethyl)*o*-	50
Ammonium, [4-(bis(*p*-(dimethylamino)phenyl) methylene)-2,5-cyclohexadien-1-xylidene] dimethyl-, chloride	50
Acetanilide, 4'-hydroxy	50
Methane, iodo-	50
2-Naphthol	50
Quinoline, 8[(4-(diethylamino)-1-methylbutyl) amino]-6-methoxy-	50
Sodium fluoride	75
Benzoic acid	500
Ethyl alcohol	500
Sulfamic acid	500
2-Pentanone, 4-methyl-	500
1,4-Naphthoquinone, 2-methyl-	500

[a] Compounds listed in order of decreasing toxicity.

Table 3.5. LD_{Lo} differences between humans and animals

Species compared	Route of administration	Percent differences				
		0	1 to 25	26 to 50	51 to 75	76 to 100
Rat	Oral				Rat[a]	Human, rat(2)[b]
	Skin			Human		
	Intravenous					Rat
Rabbit	Oral		Rabbit	Rabbit(2)[b]	Human, rabbit(3)[b]	Rabbit(5),[b] human
	Intravenous					Rabbit
Pigeon	Oral				Pigeon	Pigeon
Guinea pig	Oral			Guinea pig(2)[b]		Guinea pig
Chicken	Oral		Chicken			
Cat	Oral		Cat			
Mouse	Oral		Mouse			
Dog	Oral	1[c]			Human	Dog(7),[b] human(2)[b]
	Skin		Dog			
	Intravenous					Dog

[a] Indicates which animal of the comparison had the greatest LD_{Lo}. Position of species indicates the percent differences in the LD_{Lo} of the human and animal species being compared.

[b] Represents the number of chemicals compared where the LD_{Lo} of the species indicated was greater than the species being compared. If there is no number then only one chemical was compared.

[c] For one chemical, the human and the dog had identical LD_{Lo} values when administration was oral.

3. Only 19.5% of the comparisons showed the human LD_{Lo} value to be greater than the animal LD_{Lo} value.

On the basis of these data, it can be concluded that there is a significant difference between the human and animal acute LD_{Lo} response and that, in the majority of instances, the human showed less tolerance (reflected by lower LD_{Lo} values) to chemical insult than did the animal model.

3.3.3 Comparison of Acute Toxicity Response

The studies of Goyer (1971) and Lock and Ishmael (1978) show the effect of the acute administration of chemicals on the rat and human kidney to be similar. In his review of the effects of lead on the kidney, Goyer found that morphological and functional reactions of the kidneys of rat and man to acute lead exposure have comparable features: (1) the proximal tubular lining cells are affected, (2) intranuclear inclusion bodies are formed, and (3) there is an associated aminoaciduria. He also noted that although the impairment of mitochondrial function has not been demonstrated in man as it has in rats, the impairment may be inferred from morphological changes in human biopsy material. Lock and Ishmael in studying the acute effects of paraquat and diquat found that an oral dose of 680 micromoles/kg of either chemical would result in marked diuresis, proteinuria, and glucosuria 6 to 24 h after dosing of the male rat. In addition, histopathological kidney examination showed mild hydropic change in the proximal convoluted tubules, and renal clearance of insulin, *p*-aminohippuric acid (PAH), and *N*'-methylnicotamide (NMN) was found to be markedly reduced 2 h after dosing. Lock and Ishmael reported that these changes indicate that renal impairment is similar in rat and man.

Hexachlorophene and ethchlorvynol have also been shown to produce similar symptoms in humans and animals after acute exposure. Martinez, Boehm, and Hadfield (1974) reported the accidental oral ingestion of approximately 45 mL of hexachlorophene by a seven-year-old boy. Toxic reactions included nausea, vomiting, anorexia, diarrhea, decrease in visual acuity, blurred vision, blindness, somnolence, and disorientation, with respiratory and cardiac arrest occurring 61 h after hospital admission. Further examination revealed the following: (1) interstitial myocarditis, (2) pneumonitis and acute bronchiolitis, (3) edematous brain tissue, (4) occasional neuronal degeneration, and (5) myelin sheath disintegration and other neuronal changes. Martinez, Boehm, and Hadfield noted that these changes have been produced in experimental animals exposed to several chemicals including hexachlorophene.

In a case report of an intravenous injection of ethchlorvynol presented by Payne, Kerr, and Diaconis (1977), the individual developed acute, severe pulmonary edema accompanied by hypoxemia and acidosis. Similar toxic effects on the pulmonary vasculature were observed in rats by Payne and coworkers after administering intravenous injections of 80 mg/kg ethchlorvynol, resulting in death from acute respiratory distress.

3.3.4 Conclusions

The information in Sect. 3.3.2 concerning lowest lethal doses clearly indicates a difference between humans and animals with respect to the size of the dose that will cause death; in most cases a smaller dose was required to cause death in humans than in animals. Because of this difference, an examination of the pathology and functional changes induced by the acute exposure of humans and animals to chemicals (Sect. 3.3.3) might be expected to show corresponding differences. However, the available literature showed that, for those chemicals compared, man and animal acute toxicity was similar. This does not imply that there are no chemicals for which the human and animal response would be different, but rather indicates the scarcity of information comparing human and animal acute toxicity studies. Two possible reasons for the apparent paucity of comparable data are that (1) many acute toxicity studies performed on animals are done primarily to establish the LD_{50} and provide reference toxicity dose information for subchronic and chronic testing and (2) most acute human exposures, with the possible exception of dermal testing, occur only from accidental intake or suicides and, unless the person is admitted to a hospital soon after intake, much important data go unrecorded. Although it is not possible to satisfactorily appraise the comparative susceptibilities between man and animal with only the few chemicals used as examples in Sect. 3.3.3, the examples do support one of the major premises of toxicity research — that effects observed in animals serve as an indicator of what a probable response would be in man.

3.4 PATHOLOGY

3.4.1 Introduction

Evidence of tissue damage from the acute exposure of a test animal to a chemical would be useful information for the hazard evaluation of most chemicals, especially for those chemicals for which there is known or potential acute human exposure. The following sections consider some aspects of histopathology in acute toxicity testing of chemicals and offer some concluding remarks concerning the degree of necropsy required when evaluating the acute toxicity of a chemical.

3.4.2 Histopathology

Histopathological changes from acute exposures can be induced by a variety of compounds (some examples are given in Table 3.6), a fact not surprising since the experimental doses given are usually of such magnitude that they kill at least one-half of the test animals. The stress to the animal's system would in many cases produce lesions of various organs, if the animal lives long enough for histologic damage to occur. On the other hand, there are compounds that, when administered at doses high enough to cause death, do not show any histopathology related to the chemical. An example of this type of action was described by Robens (1979) where several strains of rats were given high doses (2000 mg/kg) of a short-chain chlorinated hydrocarbon. Several animals died as a result of the exposure, but in no animals, living or dead, was there any detectable histopathology related to the test chemical. Another example is shown by the action of *N*-2-fluorenylacetamide in mice where LD_{50} doses resulted in essentially normal tissues, as revealed by histopathological examination (Haley, Dooley, and Harmon, 1973).

Table 3.6. Examples of histopathological changes from acute exposures to chemicals

Test agent	Species	Route	Dose	Histopathology	Reference
3',4'-Dichloropropionanilide	Rat	Oral	50–700 mg/kg	Congestive hepatitis, gastroenteritis, and patchy emphysematous lung changes	Chand, 1973
Cadmium chloride	Rat	Intravenous	Not given	Lesions in endothelial clefts of small vessels in target organs such as testis and gasserian ganglion	Gabbiani et al., 1974
Dichlorvos	Dog	Intravenous and oral	2.2–22.0 mg/kg	Pulmonary changes with generalized congestion and hyperemia and cardiovascular changes	Snow, 1973
Maytansine	Rat	Subcutaneous	0.38–1.0 mg/kg	Lesions in gastrointestinal tract mucosa, thymus, spleen, bone marrow, and testis; arrest of mitotic cell division; hemorrhagic lesions in parenchymatous organs and brain; and chromatolysis and vacuolation of dorsal root ganglion cells	Mugera and Ward, 1977
Mirex	Rat and mouse	Intraperitoneal	330–700 ppm	Patchy lesions on surface of livers that extended into the liver interior several millimeters	Kendall, 1974
Ochratoxin A	Rat	Oral	15–50 mg/kg	Severe catarrhal or erosive enteritis in the duodenum and jejunum	Kanisawa et al., 1977
Aflatoxin B_1	Monkey	Oral	1–3 mg/kg	Liver damage characterized by hemorrhage, necrosis, and massive accumulation of lipid	Rao and Gehring, 1971

What information do acute toxicity tests, in particular the histopathological data, provide? Robens (1979) writes that in the development of a drug, acute toxicity studies can: (1) establish some measure of toxicity that can aid in choosing doses for studies of longer duration, (2) help to assess whether the drug has efficacy in the non-toxic range, (3) enable certain conclusions to be made regarding mechanism of action, if the animals are carefully observed following treatment and then necropsied, and (4) answer the question of how much difference exists between dosages that seem to be safe and those that are known to be highly toxic. Histopathological information derived from acute toxicity tests can also aid physicians confronted with cases of accidental poisoning or attempted suicides by providing information on chemical effects parameters such as target organs

Histologic evidence of damage to various organ systems as a result of acute exposure often provides evidence that permits prediction as to whether the organ system will likely be similarly affected after chronic dosing, but this is not always the case. Three studies that illustrate that the results from acute toxicity testing cannot always be used to predict the results of chronic toxicity testing are reported by Kanisawa et al. (1977), Diamond and Sleight (1972), and Pennarola, Balletta, and DiPaolo (1969). Kanisawa et al. (1977) found that oral administration of near-lethal single doses of ochratoxin A (a mycotoxin) to rats did not result in renal damage, but several consecutive daily doses produced massive acidophilic degeneration with necrosis and desquamation of epithelium in the proximal tubules. Second, in a study of the acute and subchronic effects of methylmercury dicyandiamide in the rat, Diamond and Sleight (1972) reported that atrophy of the granular cells of the cerebellum and hepatocytes was present only in those animals subjected to repeated exposures. Third, in the research of Pennarola, Balletta, and DiPaolo (1969), acute exposure of the rabbit to parathion resulted primarily in hyperemia of the internal organs with pulmonary emphysema and edema, whereas chronic exposure produced lesions of a moderate nature; these lesions were more pronounced in the excretory organs because of the longer contact of these organs with parathion and its metabolites.

3.4.3 Conclusions

Many acute toxicity studies are designed to give a quantitative measure of acute toxicity (LD_{50}) for comparison with other substances and/or dose-ranging guidance for subchronic or chronic tests; thus, the necessity of having detailed histological data is minimal. Those toxicity studies, however, that seek to completely characterize the acute toxicity of a chemical would of course necessitate necropsy. Because histopathological changes that occur from acute exposures to chemicals may not be the same as those changes that result from longer exposures and because many acute exposures cause death relatively quickly (especially at LD_{50} levels—see Table 3.7) and thus may not cause tissue damage, the necessity of performing histopathological examinations for each chemical tested for acute toxicity can be questioned. However, because some chemicals do have the potential for acute exposure to humans and because some knowledge of such tissue changes would be useful (e.g., for comparison with other chemicals tested for acute toxicity), gross necropsy seems warranted for all animals when significant signs of toxicity are observed, with consideration of histopathology for organs showing evidence of gross pathology in animals surviving 24 h or more. This view is supported by Zbinden (1973), who states that histopathological examination of the organs is not recommended as a routine procedure because (1) the organ changes of animals dying from acute overdosage are so ambiguous that an intelligent assessment would be difficult and (2) the information likely to be gained over that furnished by gross necropsy would only occasionally justify the additional technical effort.

3.5 OBSERVATION PERIOD

3.5.1 Introduction

The LD_{50} is the dose that will result in the death of one-half of the test animals to which it is administered. Inherent in this definition is an observation period of some finite length; however, after reviewing several papers that describe the acute toxicities of various chemicals, it is obvious that the observation periods are not always the

Table III-[illegible]. Observation periods

Chemical	Species	Route	LD50 (mg/kg)	Observation period (days)	Time death reported (days)	Reference
Methylmercury dicyandiamide	Rat	Intraperitoneal	[illegible]	[illegible]		[illegible], 19[illegible]
Ochratoxin A	Rat	Oral	[illegible]	[illegible]		[illegible] et al., [illegible]
Aflatoxin B_1	Monkey	Oral	[illegible]	[illegible]	[illegible]	[illegible] and [illegible], [illegible]
Maytansine	Rat	Intravenous	[illegible]	[illegible]	[illegible]	[illegible] and [illegible], [illegible]
Proximphan	Mice	Oral	[illegible]	[illegible]		[illegible], [illegible], and [illegible], 19[illegible]
Guanethidine	Rat	Oral	[illegible]	[illegible]		[illegible] et al., [illegible]
N-Methyl-N-(1-napthyl)fluoro-acetamide	Mice	Oral	[illegible]	[illegible]	[illegible]	[illegible] et al., [illegible]
Probucol	Rat	Oral	[illegible]			[illegible], [illegible], and [illegible], 197[illegible]
Phenoxarsine oxide	Guinea pig	Oral	[illegible]	[illegible]	[illegible]	[illegible], 197[illegible]
Pentachlorophenol	Mice	Oral	[illegible]			[illegible] and [illegible], [illegible]
Nefopam	Dog	Intravenous	[illegible]	[illegible]	[illegible]	[illegible], [illegible], and [illegible], 197[illegible]
Phenazine-5,10-dioxide	Rat	Oral	≈ 600	[illegible]		[illegible] et al., 197[illegible]
Cyclopiazonic acid	Rat	Oral	[illegible] (male) [illegible] (female)	[illegible]	[illegible]	[illegible], 197[illegible]
Aroclor 1254	Rat	Oral	≈ [illegible]	[illegible]		[illegible], [illegible], and [illegible], 197[illegible]
Hydralazine	Rat	Oral	[illegible] (male) [illegible] (female)	[illegible]	[illegible]	[illegible] et al., 197[illegible]

Table 3.7 (continued)

Chemical	Species	Route	LD_{50} (mg/kg)	Observation period (days)	Time deaths occurred (days)	Reference
2,3-Dihydro-9H-isoxasolo-(3,2-b)quinazolin-9-one	Dog	Oral	700 ± 110	14	1	Banerjee et al., 1977[a]
Oxepinac	Rat	Oral	136	7	6	Nomura et al., 1978[c]
Photomirex	Rat	Oral	150 to 200 (male)	28		Hallett et al., 1978[c]
Ethylidene gyromitrin	Rabbit	Oral	70	3.5	3.5	Makinen, Kreula, and Krauppi, 1977[c]
Sulfotep	Mice Mice	Oral Intraperitoneal	29.4 15.2	12	1	Kimmerle and Klimmer, 1974[c]
Benzene	Rat	Intraperitoneal	2 940	14	<1	Drew and Fouts, 1974[a]
Cadmium	Rat	Oral	225	14		Kotsonis and Klassen, 1977[a]
Di-2-ethylhexyl phthalate	Mice	Intraperitoneal	37 770	7		Lawrence et al., 1975[a]
Dichlorodiphenyl-trichloroethane	Mice	Oral	237	10		Tomatis et al., 1972[b]
Ethanol	Rat	Oral	10 600	1		Wiberg, Trenholm, and Coldwell, 1970[c]
	Mice	Intravenous	6.08	7		Beliles, 1972[a]
Clindamycin hydrochloride	Mice	Oral	2 618	7		Gray et al., 1972[a]
Dipterex	Rat	Oral	649	7	5	Edson and Noakes, 1960[c]
Dimethyl terephthalate	Rat	Intraperitoneal	3 900	14	2	Krasavage, Yanno, and Terhaar, 1973[a]
Peroxyacetyl nitrate	Rat	Inhalation	95 ppm (LC_{50})	14	1	Kruysse et al., 1977[b]
Chloropyrifos	Rat (various strains)	Oral	118 to 245		-	McCollister et al., 1974[a]

Table 3.7 (continued)

Chemical	Species	Route	LD_{50} (mg/kg)	Observation period (days)	Time deaths occurred (days)	Reference
trans-2-Hexenal	Rat (male)	Oral	780	14	2	Gaunt et al., 1971[a]
Thiabendazole hydrochloride	Rat	Oral	3 100	10		Robinson, Stoerk, and Graessle, 1965[a]
Cefazolin	Mice	Oral	>10 000 (no deaths at 10 000)	7		Birkhead, Briggs, and Saunders, 1973[a]
SMA 1440-H resin	Rat (female)	Oral	>20 but <22	14	Within 3 days	Winek and Burgun, 1977[a]
Alromine RU 100	Rat	Oral	>3 200	10		Hunter and Stevenson, 1967[b]
Fenoterol HBr	Rat (male)	Oral	2 300	7	1	Kast et al., 1975a[b]
Ethylene dibromide	Rat (male)	Oral	146	14		Rowe et al., 1952[a]
Hexachlorophene	Rat (male)	Oral	69.2	3	3	Nakaue, Dost, and Buhler, 1973[a]
A204	Rat	Oral	7.62	7–14	3	Worth et al., 1970[a]
Cadmium ions	Rat	Oral	130 to 180	14		Lorke, 1978[b]
1,1,3,3-Tetraethoxypropane	Rat	Oral	1 610	14	4	Crawford et al., 1965[a]
2-Methylaniline	Rat	Oral	900	14		Jacobson, 1972[a]

[a] U.S. paper.

[b] Foreign paper.

same. This section primarily discusses the length of the observation period of test animals following acute exposure to a chemical.

3.5.2 Purpose of Observation Period

The purpose of the observation period is to allow time for manifestation of the toxic effect, which in determination of the LD_{50} is either survival or death. The observation period thus needs to be of sufficient duration that the metabolism and elimination of the test substance from the test animal is near completion when the LD_{50} is determined. The importance of this is revealed by the fact that many compounds have different rates of elimination. An example is that of three tetrachlorophenol compounds: 2,3,5,6-, 2,3,4,6-, and 2,3,4,5-tetrachlorophenol. Ahlborg and Larsson (1978) observed that following intraperitoneal injection to rats, 2,3,5,6-tetrachlorophenol was eliminated within 24 h and 2,3 4,6-tetrachlorophenol within 43 h, but only 60% of 2,3,4,5-tetrachlorophenol was eliminated and subsequently recovered in the urine within 72 h.

The observation period following acute exposure must also be of sufficient length to allow for the fact that different routes of administration can result in the toxic effect of a chemical being manifested at different times. In a study by Nomura et al. (1978), mice that received a lethal dose of oxepinac, an antiinflammatory drug, either intraperitoneally or subcutaneously, exhibited convulsion and dyspnea, with most deaths occurring within 24 h, whereas mice that received an oral lethal dose showed anorexia, emaciation, and bloody feces with deaths 2 to 6 days postadministration.

3.5.3 Length of Observation Period

Table 3.7 summarizes reported data from 43 studies. Examination of this information shows that 12 studies had an observation period of 7 days and 18 studies had an observation period of 14 days, with the remaining 13 studies having observation periods ranging from 3 to 28 days.

The *Guidelines for Carcinogen Bioassay in Small Rodents* (Sontag, Page, and Saffiotti, 1976) and guidelines proposed by the U.S. Environmental Protection Agency (1978) for registering pesticides in the United

States recommend at least a 14-day observation period. This 14-day period is thus the choice of some U.S. regulatory and chemical testing agencies. It is not surprising that many toxicological researchers in the United States, many of whom contribute to the formulation of these agencies' guidelines, also adhere to this 14-day period. Table 3.7 shows that 14 of 24 studies by U.S. researchers used a 14-day observation period.

In addition to showing variation in observation periods, the information in Table 3.7 also raises the question of which length is the most desirable. As previously mentioned (Sect. 3.5.2), the observation period must be of sufficient duration to allow for manifestation of the toxic effect; however, the time period cannot be so long that the animal's death could be attributed to factors other than the primary effect of the chemical being tested. Further examination of some of the papers cited in Table 3.7 yields some interesting facts with regard to time of death following acute administration. Rao and Gehring (1971) subjected male cynomolgus monkeys (*Macaca irus*) to acute oral doses of aflatoxin B_1 and found that all deaths occurred within the first 4 days of the 14-day observation period. Similar results were seen when 2,3-dihydro-9H-isoxazolo(2,3-b-quinazolin-9-one was administered orally to dogs (Banerjee et al., 1977). Within 24 h after dosing, 4 out of 4 dogs died at at dose of 1150 mg/kg and 2 out of 4 dogs died at a dose of 750 mg/kg. The LD_{50}s of mice and rats were also determined in this study, but no indication is provided as to when the animals died within the 2-week observation period. Research with budralazine (Onodera et al., 1978) and nefopam (Case, Smith, and Nelson, 1975) also indicated that death from acute exposure may occur within a few hours or days. The LD_{50} studies with budralazine showed that most deaths occurred within 48 h, with all survivors appearing normal 72 h after dosing, whereas death of mice, rats, and dogs occurred even more quickly with nefopam – 1 to 10 min after intravenous injection, 15 to 30 min after intramuscular injection, and 30 to 120 min after oral dosage.

Still further evidence of short survival time following acute administration is provided by the works of Kimmerle and Klimmer (1974) and Diamond and Sleight (1972). Kimmerle and Klimmer observed that mice, rats, rabbits, cats, dogs, and hens exposed to sulfotep, either orally or intraperitoneally, died within 24 h and that survivors recovered within 1 to 4 days. Diamond and Sleight (1972) discovered that, if rats acutely exposed to methylmercury dicyandiamide were going to die, death would occur within 72 to 78 h.

3.5.4 Conclusions

Although only a relatively small number of papers were reviewed, it seems evident that many U.S. researchers employ the 14-day observation period following acute administration of a chemical, whereas many non-U.S. researchers appear to use observation periods of various lengths. It is also evident that in several instances test animals died well within the specified observation period. Therefore, the 14-day observation period suggested by most regulatory agencies seems sufficient for the determination of the LD_{50} for most chemicals.

REFERENCES FOR SECTION 3

Ahlborg, U. G., and K. Larsson. 1978. Metabolism of Tetrachlorophenols in the Rat. Arch. Toxicol. 40:63-74.

Ballantyne, B. 1978. The Comparative Short-Term Mammalian Toxicology of Phenarsazine Oxide and Phenoxarsine Oxide. Toxicology 10:341-361.

Banerjee, B. N., R. D. Sofia, N. J. Ivins, and B. J. Ludwig. 1977. Toxicological Investigation of 2,3-Dihydro-9H-isoxazolo[3,2-*b*]quinazolin-9-one (W-2429): Acute and Subacute Toxicity in Mice, Rats, and Dogs. Drug Res. 27(1):793-801.

Beliles, R. P. 1972. The Influence of Pregnancy on the Acute Toxicity of Various Compounds in Mice. Toxicol. Appl. Pharmacol. 23:537-540.

Birkhead, H. A., G. B. Briggs, and L. Z. Saunders. 1973. Toxicity of Cefazolin in Animals. J. Infect. Dis. 128 (Suppl.):S379-S381.

Bruckner, J. V., K. L. Khanna, and H. H. Cornish. 1973. Biological Responses of the Rat to Polychlorinated Biphenyls. Toxicol. Appl. Pharmacol. 24:434-448.

Case, M. T., J. K. Smith, and R. A. Nelson. 1975. Reproductive, Acute, and Subacute Toxicity Studies with Nefopam in Laboratory Animals. Toxicol. Appl. Pharmacol. 33:46-51.

Chand, N. 1973. Acute Toxicity of 3',4'-Dichloropropionanilide in Rats. Indian Vet. J. 50(11):1122-1125.

Crawford, D. L., R. O. Sinnhuber, F. M. Stout, J. E. Oldfield, and J. Kaufmes. 1965. Acute Toxicity of Malonaldehyde. Toxicol. Appl. Pharmacol. 7:826-832.

Diamond, S. S., and S. D. Sleight. 1972. Acute and Subchronic Methylmercury Toxicosis in the Rat. Toxicol. Appl. Pharmacol. 23:197-207.

Drew, R. T., and J. R. Fouts. 1974. The Lack of Effects of Pretreatment with Phenobarbital and Chlorpromazine on the Acute Toxicity of Benzene in Rats. Toxicol. Appl. Pharmacol. 27:183-193.

Edson, E. F., and D. N. Noakes. 1960. The Comparative Toxicity of Six Organophosphorus Insecticides in the Rat. Toxicol. Appl. Pharmacol. 2:523-539.

Food Safety Council. 1978. Proposed System for Food Safety Assessment. pp. 5, 29-33.

Gabbiani, G., M. C. Badonnel, S. M. Mathewson, and G. B. Ryan. 1974. Acute Cadmium Intoxication: Early Selective Lesions of Endothelial Clefts. Lab. Invest. 30(6):686-695.

Gaines, T. B. 1960. The Acute Toxicity of Pesticides to Rats. Toxicol. Appl. Pharmacol. 2:88-99.

Gaines, T. B. 1969. Acute Toxicity of Pesticides. Toxicol. Appl. Pharmacol. 14:515-534.

Gaunt, I. F., J. Colley, M. Wright, M. Creasey, P. Grasso, and S. D. Gangolli. 1971. Acute and Short-Term Toxicity Studies on Trans-2-Hexenal. Food Cosmet. Toxicol. 9:775-786.

Goyer, R. A. 1971. Lead and the Kidney. Curr. Top. Pathol. 55:147-176.

Gray, J. E., R. N. Weaver, J. A. Bollert, and E. S. Feenstra. 1972. The Oral Toxicity of Clindamycin in Laboratory Animals. Toxicol. Appl. Pharmacol. 21:516-531.

Haley, T. J., K. L. Dooley, and J. R. Harmon. 1973. Acute Oral Toxicity of *N*-2-Fluorenylacetamide (2-FAA) in Several Strains of Mice (abstract). Proc. Soc. Exp. Biol. Med. 143(4):1117-1119.

Hallett, D. J., K. S. Khera, D. R. Stoltz, I. Chu, D. C. Villeneuve, and G. Trivett. 1978. Photomirex: Synthesis and Assessment of Acute Toxicity, Tissue Distribution, and Mutagenicity. J. Agric. Food Chem. 26(2):388-391.

Hartnagel, R. E., B. M. Phillips, E. H. Fonseca, and R. L. Kowalski. 1976. The Acute and Target Organ Toxicity of 1-Methyl-3-keto-4-phenylquinuclidinium Bromide (MA 540) and Guanethidine in the Rat and Dog. Drug Res. 26(9):1671-1672.

Hashimoto, Y., T. Makita, H. Miyata, T. Noguchi, and G. Ohta. 1968. Acute and Subchronic Toxicity of a New Fluorine Pesticide, *N*-Methyl-*N*-(1-naphthyl) Fluoracetamide. Toxicol. Appl. Pharmacol. 12(3):536-547.

Haynes, W. J. 1975. General Principles: Dosage and Other Factors Influencing Toxicity. In: Toxicity of Pesticides. Williams and Wilkins Company, Baltimore. pp. 37-100.

Hunter, C. G., and D. E. Stevenson. 1967. Acute and Subacute Oral Toxicity of Alromine RU 100 in Rats. Food Cosmet. Toxicol. 5:491-496.

Jacobson, K. H. 1972. Acute Oral Toxicity of Mono- and Di-Alkyl Ring-Substituted Derivatives of Aniline. Toxicol. Appl. Pharmacol. 22:153-154.

Kanisawa, M., S. Suzuki, Y. Kozuka, and M. Yamazaki. 1977. Histopathological Studies on the Toxicity of Ochratoxin A in Rats: I. Acute Oral Toxicity. Toxicol. Appl. Pharmacol. 42(1):55-64.

Kast, A., Y. Tsunenari, M. Honma, J. Nishikawa, T. Shibata, and M. Torii. 1975*a*. Acute, Subacute and Chronic Toxicity Studies of the Beta-Sympathomimetic, Fenoterol HBr on Rats, Mice and Rabbits. Oyo Yakuri 10(1):45-71.

Kast, A., Y. Tsunenari, M. Honma, J. Nishikawa, T. Shibata, and M. Torii. 1975*b*. Acute, Subacute and Chronic Toxicity Studies of an Amino-Halogen-Substituted Benzylamine (Fominoben) in Rats and Mice. Oyo Yakuri 10(1):31-43.

Kendall, M. W. 1974. Acute Histopathologic Alterations Induced in Livers of Rat, Mouse, and Quail by the Fire-Ant Poison, Mirex. Anat. Rec. 178: 388.

Kimmerle, G., and O. R. Klimmer. 1974. Acute and Subchronic Toxicity of Sulfotep. Arch. Toxicol. 33:1-16.

Koeda, T., M. Odaki, H. Sasaki, M. Yokota, T. Niizato, H. Watanabe, H. Kawaoto, and T. Watanuki. 1976. Toxicological Studies on Phenazine-5*N*-Oxide in Rats. Oyo Yakuri 12(3):483-499.

Kotsonis, F. N., and C. D. Klaassen. 1977. Toxicity and Distribution of Cadmium Administered to Rats at Sublethal Doses. Toxicol. Appl. Pharmacol. 41:667-680.

Krasavage, W. J., F. J. Yanno, and C. J. Terharr. 1973. Dimethyl Terephthalate (DMT): Acute Toxicity, Subacute Feeding, and Inhalation Studies in Male Rats. Amer. Ind. Hyg. Assoc. J. 34(1):455-462.

Kruysse, A., V. J. Feron, H. R. Immel, B. J. Spit, and G. J. Von Esch. 1977. Short-Term Inhalation Toxicity Studies with Peroxyacetyl Nitrate in Rats. Toxicology 8:231-249.

Lawrence, W. H., M. Malik, J. E. Turner, A. K. Singh, and J. Autian. 1975. A Toxicological Investigation of Some Acute, Short-Term, and Chronic Effects of Administering Di-2-Ethylhexyl Phthalate (DEHP) and other Phthalate Esters. Environ. Res. 9:1-11.

Lewerenz, H. J., G. Lewerenz, and R. Plass. 1970. Acute (Mouse and Rat) and Subacute (Rat) Toxicity of the Herbicide, Proximpham. Food Cosmet. Toxicol. 8(5):517-526.

Lock, E. A., and J. Ishmael. 1978. The Effects of Paraquat and Diquat on Rat Kidney (abstract). Toxicol. Appl. Pharmacol. 45(1):227.

Lorke, D. 1978. New Studies on Cadmium Toxicology. Proc. First Int. Cadmium Conf. pp. 175-180.

Makinen, S. M., M. Kreula, and M. Kauppi. 1977. Acute Oral Toxicity of Ethylene Gyromitrin in Rabbits, Rats and Chickens. Food Cosmet. Toxicol. 15(6):575-578.

Martinez, A. J., R. Boehm, and M. G. Hadfield. 1974. Acute Hexachlorophen Encephalopathy: Clinico-Neuropathological Correlation. Acta. Neuropathol. 28(2):93-104.

McCollister, S. B., R. J. Kociba, C. G. Humiston, D. D. McCollister, and P. J. Gehring. 1974. Studies of the Acute and Long-Term Oral Toxicity of Chlorpyrifos (*O*,*O*-Diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate). Food Cosmet. Toxicol. 12:45-61.

Molello, J. A., C. G. Gerbig, and V. B. Robinson. 1973. Toxicity of [4,4'-(Isopropylidenedithio)bis(2,6-di-t-butylphenol)], Probucol, in Mice, Rats, Dogs and Monkeys: Demonstration of a Species-Specific Phenomenon. Toxicol. Appl. Pharmacol. 24:590-593.

Mugera, G. M., and J. M. Ward. 1977. Acute Toxicity of Maytansine in F344 Rats. Cancer Treat. Rep. 61(7):1333-1338.

Nakaue, H. S., F. N. Dost, and D. R. Buhler. 1973. Studies on the Toxicity of Hexachlorophene in the Rat. Toxicol. Appl. Pharmacol. 24:239-249.

National Academy of Sciences. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. Printing and Publishing Office, Washington, D.C. 130 pp.

National Institute for Occupational Safety and Health. 1977. Registry of Toxic Effects of Chemical Substances, Vol. 2. E. J. Fairchild, R. J. Lewis, and R. L. Tatken, eds. 987 pp.

Nomura, M., T. Onodera, M. Kato, A. Yamada, H. Ogawa, and T. Akimoto. 1978. Acute, Subacute, and Chronic Toxicity of Oxepinac. Arzneim.-Forsch. 28(3):445-451.

Onodera, T., S. Takayama, A. Yamada, Y. Ono, and T. Akimoto. 1978. Toxicological Studies of 1-[2-(1,3-Dimethyl-2-butenylidene)hydrazino]-phthalazine, a New Anti-hypertensitive Drug, in Mice and Rats. Toxicol. Appl. Pharmacol. 44(3):431-439.

Payne, C. B., Jr., H. D. Kerr, and J. N. Diaconis. 1977. Pathophysiologic Effects of Intravenous Ethchlorvynol (Placidyl) in Man Following Acute Pulmonary Edema. Md. State Med. J. 26(6):68-70.

Pennarola, R., Balletta, and R. DiPaolo. 1969. Histopathological Findings in Acute and Chronic Experimental Intoxication with Parathion (abstract). Folia Med. 52(2):118-129.

Purchase, I.F.H. 1971. The Acute Toxicity of the Mycotoxin Cyclopiazonic Acid to Rats. Toxicol. Appl. Pharmacol. 18:114-123.

Rao, K. S., and P. J. Gehring. 1971. Acute Toxicity of Aflatoxin B_1 in Monkeys. Toxicol. Appl. Pharmacol. 19:169-175.

Robens, J. F. 1979. Assessment of the Safety of Drugs for Animals. Vet. Hum. Toxicol. 21(1):12-23.

Robinson, H. J., H. C. Stoerk, and O. E. Graessle. 1965. Studies on the Toxicologic and Pharmacologic Properties of Thiabendazole. Toxicol. Appl. Pharmacol. 7:53-63.

Rowe, V. K., H. C. Spencer, D. D. McCollister, R. L. Hollingsworth, and E. M. Adams. 1952. Toxicity of Ethylene Dibromide Determined on Experimental Animals. Arch. Ind. Hyg. Occup. Med. 6:158-173.

Snow, D. H. 1973. The Acute Toxicity of Dichlorvos in the Dog: 2. Pathology. Aust. Vet. J. 49(3):120-125.

Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. National Cancer Institute, NCI-CG-TR-1. 65 pp.

Tomatis, L., Y. Turusov, N. Day, and R. T. Charles. 1972. The Effect of Long-Term Exposure to DDT on CF-1 Mice. Int. J. Cancer 10(3):489-506.

U.S. Environmental Protection Agency. 1978. CFR 40 No. 163.81.

Wiberg, G. S., H. L. Trenholm, and B. B. Coldwell. 1970. Increased Ethanol Toxicity in Old Rats: Changes in LD$_{50}$, In Vivo and In Vitro Metabolism, and Liver Dehydrogenase Activity. Toxicol. Appl. Pharmacol. 16:718-727.

Winek, C. L., and J. J. Burgun. 1977. Acute and Subacute Toxicology and Safety Evaluation of SMA 1440-H Resin. Clin. Toxicol. 10(2):255-260.

Worth, H. M., D. B. Meyers, W. R. Gibson, and G. C. Todd. 1970. Acute and Subacute Toxicity of A204. In: Antimicrobial Agents and Chemotherapy - 1970. pp. 357-360.

Zbinden, G. 1973. Acute Toxicity. In: Progress in Toxicology. Springer-Verlag, Inc., New York. pp. 23-27.

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4. SUBCHRONIC TEST DESIGN

4.1 INTRODUCTION

The standard protocol for toxicity testing of chemicals generally proceeds from single-dose tests of short duration (acute) to repeated-dose tests of long duration (chronic). Part of this procedure is a repeated-dose study of intermediate duration, referred to as a subchronic or subacute test. The subchronic test provides information on the toxic effects of chemicals that are likely to occur from repeated exposures over a limited time period. Currently 14-, 28-, and 90-day studies are commonly used; however, a review of the literature indicates the duration of the study may vary widely.

The first recorded definition of a subchronic test was as follows: a repeated exposure test with a duration of one-tenth the expected life span of the experimental animal used (Food and Agriculture Organization/World Health Organization Technical Report, 1958). Boyd (1961) thought that for the rat this meant a duration of approximately 100 days. A 13-week test with four dosage levels was the definition given for subacute tests by Abrams, Zbinden, and Bagdon (1965). Loomis (1974) described the repeated exposure test as a "prolonged test" with daily exposure for about 3 months. Guarino (1979) stated that traditionally three terms (*short-term toxicity*, *subchronic*, or *prolonged*) are used interchangeably to designate a 90-day test. Many similar definitions, based upon a repeated dose given over a period of a few days to 3 months, have appeared in the literature, including some by expert committees (National Academy of Sciences, 1975; 1977). The culmination of these efforts was the definition and guidelines of the U.S. Environmental Protection Agency (EPA) pesticide programs (*Federal Register*, 1978). This guideline set standards for subchronic tests covering dosage levels, routes of exposure, number of species, number of test animals, type and degree of pathology, hematology, biochemical and function evaluations, etc. The choice of *subchronic* as the term of preference is followed in this text and replaces all other terms. "However, the important thing is not the choice of definition of a particular fraction of the life span but the selection of a testing interval that is as short as practicable and yet will give meaningful information about the effect of absorbing the toxicant during an entire lifetime" (Hayes, 1975). In addition to summarizing the definition of a subchronic test, this quotation describes the purpose of this chapter, which is to evaluate the currently employed subchronic tests to determine whether they provide sufficient information that makes longer-term tests unnecessary. The section does not consider a comparison of time versus effect for dermal, ocular, or inhalation toxicity and does not consider carcinogenic effect.

The two primary functions of subchronic tests, as they are currently used, are (1) to assess toxic effects from repeated exposure over a relatively short time so as to give maximum and minimum effect levels with safety margins plus a prediction of possible effects from longer exposures and (2) to determine the appropriate dose levels to be used if longer exposures are planned (Benitz, 1970; Peck, 1968). The purpose of this chapter is to evaluate the first of these two functions. To fulfill this first function, a battery of biochemical, hematological, and pathological tests is performed. These tests provide data upon which more selective evaluations can be based. The target organ data are especially useful. The value of such data has been recognized for several decades, and, as stated by Barnes and Denz (1954), "It is in fact doubtful whether it is worth proceeding beyond the subacute test." The second function is an elaboration of the acute evaluation of effective doses and should give maximum and minimum effect levels with safety margins. The role of the subchronic test as a preliminary evaluation of appropriate dose levels for use in longer test designs (such as a carcinogenicity bioassay) is evaluated in a separate review by Prejean (Appendix A). However, as the time and cost requirements of toxicity testing increase, so does the value of subchronic tests as reasonable evaluations of chronic effects. If a more efficient subchronic test design can be constructed, the need for longer, more costly chronic studies may be reduced.

The standard or typical experimental design for a subchronic test has often been discussed in the literature. Recent examples include a discussion of procedures for the preclinical toxicologic evaluation of cancer chemotherapy agents (Prieur et al., 1973); a proposed system for food safety assessments (Food Safety Council, 1978); a review of principles and methods for evaluating chemical toxicities (World Health Organization, 1978); and proposed designs being developed by the Interagency Regulatory Liaison Group (IRGL) and Organization for Economic Cooperation and Development (OECD). A representative sample of these test designs is included in Table 4.1. Overall these systems are quite similar. Selected areas of these standard designs will be reviewed in this document to determine if modifications are possible. Thus, the designs in Table 4.1 represent the baseline system for which changes will be suggested.

4.2 SPECIES

4.2.1 Introduction

The debate over which and how many species to employ in subchronic toxicity tests has been a part of toxicology for many years. Discussions generally center on: (1) what species best represents human responses, (2) what is the most sensitive species for a given test or effect, and (3) what is the value of rodent and nonrodent combinations. To evaluate these problems, this review will examine literature reviews and discussions concerning species applicability and data from species comparison studies.

4.2.2 Discussions in Literature Reviews Concerning Species Suitability

Barnes and Denz (1954) in their review of chronic tests discussed the choice of test animals. They ranked the rat as the "first choice" because of economic and scientific factors. The choice of a second, nonrodent species should be based on the similarity to human responses. Both the monkey and dog have disadvantages, particularly if the monkeys are imported or the dogs are mongrel breeds. Further, Barnes and Denz questioned how often these two species added to the information gained by the rat. Only if inhalation studies (monkey) or large tissue/blood

Table 4.1. Experimental design for recently proposed subchronic oral toxicity tests

Agency	Species	Sex	Age at start	Number of animals per group (sex per dose)	Duration	Number of doses	Dose levels
Federal Insecticide, Fungicide, and Rodenticide Act (Toxic Substances Control Act) guidelines, 1978-1979[a]	Two (rat and nonrodent)	Both	Rats: 6 weeks Dogs: 4 to 6 months	Rats: 20 Dogs: 4	Rodents: 90 days Dogs: 6 months	3[b]	Highest: effect level, but not more than 10% mortality Lowest: no evidence of toxicity
Interagency Regulatory Liaison Group, 1979[a]	Two (rat and dog)	Both	Rats: 6 weeks Dogs: 4 to 6 months	Rats: 20 [illegible] if 6 months, 10 [illegible] Dogs: [illegible]	Generally 90 days; shorter for special situations	3	Highest: toxic level, but not excess mortality Lowest: no evidence of toxicity
World Health Organization, 1979	Two (rodent and nonrodent)	Both	Rats: just after weaning	10	10% life span or [illegible] months	3	Highest: distinct toxic level Lowest: no detectable toxic reaction
Food Safety Council, 1978	Two (rodent and nonrodent)	Both	(Not indicated)	Rodents: [illegible] Nonrodents: [illegible]	Rodents: 90 days [illegible] Nonrodents: 1 year	[illegible]	Highest: clearly toxic, but not lethal Lowest: no observable adverse effect, but a reasonably large multiple of estimated human daily intake

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[a] Proposed or unofficial guidelines.

[b] Must have control groups also.

Source: Adapted from Page, 1979.

samples (dog) are needed, do these species add significant data. They concluded that the use of a second species should be flexible, with the toxicologist choosing the best species, depending on the chemical or test design requirements.

In later discussions more emphasis is placed on the relationship between the test species and the human response rather than on economic or logistic variables. A good example of this change is evident in a discussion of inhalation tests (Roe, 1968), in which specific comparisons of the respiratory systems of several laboratory species are made. By examining particle deposition patterns and mucous secretion mechanisms, Roe dismissed the mouse, guinea pig, rabbit, and cat as suitable test animals. On the basis of anatomical considerations, monkeys and dogs resemble man more closely than do other species, and Roe recommended these for "small-scale, short-term experiments." The value of the rat apparently lies between these two groups, because it is more similar to man in anatomy and response than the first group and of a more manageable size and more certain genetic makeup than the dog or monkey. Thus, the importance of similarity to the human response in species selection is elevated in the present discussion to at least the same level as economic and other practical considerations.

In a later review of chronic toxicity, Benitz (1970) documented the frequency of use for various species and the reasons behind the choices. He gave the following percentages for the use of each species based on 134 studies of 1 month or longer duration (these studies appeared in *Toxicology and Applied Pharmacology* between 1959 and 1966): rat, 43.3%; dog, 38.1%; monkey, 6.7%; mouse, 3.7%; and rabbit, 3.0%. The emphasis on the rat and dog is supported in a report by Bushby, Lechat, and Santarato (1966) in which 71.7% of all toxicity testing laboratories used the dog and rat and only 16% used the rat, dog, and monkey. Benitz attributed this to investigator familiarity with the rat and dog as test species rather than to these species' superiority in sensitivity or comparability to human response patterns.

Following the method of Pinkel (1956), several authors have evaluated species suitability in anticancer drug studies by using doses based on milligrams per square meter (surface area) instead of milligrams per kilogram (body weight). This technique is useful because surface area correlates better with metabolic rate than body weight does. Therefore, by [illegible]ing doses on a surface area basis the researcher obtains tighter control of the dosage variable, allowing a truer evaluation of species differences. The results obtained when dosage sensitivity was compared in this manner suggested that in most cases the monkey, the rat, and the dog all give similar results (Freireich et al., 1966). However, in such comparisons between the dog and monkey using the maximum tolerated dose, Homan (1972) found that the dog was generally more sensitive and should be preferred over the monkey.

In an evaluation of the predictive value of test species for the metabolism of drugs, Smith (1979) compared the dog, monkey, and man. In contrast to Homan (1972), Smith preferred the monkey. By discussing and rating metabolic fate in 34 compounds for these species (Table 4.2), Smith concluded that "(1) for a large group of relatively dissimilar compounds the rhesus monkey is a significantly better predictor of metabolic fate in man than either the dog or the rat and (2) that there is little to choose between the latter two species, in both cases the correspondence is poor for over half the compounds regardless of their chemical classification."

Zbinden (1963) discussed species selection in evaluations of drug toxicities. He favored the use of the dog and the rat, with additional use of the monkey if confronted by unexplained toxic effects. However,

Table 4.2. Ranking of various animal models as predictors of metabolic fate in man

Rating	Species					
	Rat		Dog		Monkey	
	Number of compounds	Percent	Number of compounds	Percent	Number of compounds	Percent
Good	5	14	6	18	22	65
Fair	7	21	8	23	9	26
Poor	19	53	20	59	3	9
No data	3	12				
Total	34	100	34	100	34	100

Source: Adapted from Smith, 1979. Reprinted with permission of the publisher.

the key factor is metabolic similarity to man. Since absorption, penetration, and distribution processes are similar in most species, the primary differences between species are due to variations in biotransformation pathways and in rates of inactivation. The final selection of test species should reflect the species' similarity to man in metabolizing the chemical under study.

In another review of drug safety evaluations, Peck (1968) stated that the basis for experimental species selection should be a metabolic similarity to man. However, as he pointed out, the human metabolic pattern is generally unknown for new drugs. His final recommendation was to include several species in drug tests in order to protect against unknown metabolic variations.

However, Boyd (1968), in discussing species selection in his review of drug testing, did not base the choice on metabolic similarity alone. He felt another factor of equal importance is the ability to vomit (which parallels the human protective reflex). Boyd suggested the use of a species that has vomiting capabilities (recommending the dog) and one nonvomiting species that is metabolically similar to man. Although Boyd discussed only the vomiting ability of the test species, this factor is representative of many such physiological traits. His conclusions can be expanded to include the recommendation that the test species should have a physiological functional pattern that is as similar as possible to the human pattern for the specific test chemical. If such information is not known, then "use as many species as may be possible."

This reliance on several species is recommended again by McNamara (1976), although he did point out that many researchers feel comfortable using only the rat. McNamara cited Hebold (1972) regarding the regulations of six countries and three international policy-setting bodies requiring the use of the rat, dog, and rabbit, or monkey as test species. Hayes (1967a) also feels the need exists to use several species in studies of pesticide toxicity. This is based on both the general variability between animal and human responses and the importance of the species selected in determining toxic results (second only to dose levels employed). Guarino (1979), in his discussion of drug development programs, saw a definite value in using many species. He recommended the use of "(1) mice and rats for experimental therapeutic data; (2) dogs, monkeys, and mice for toxicologic studies; and (3) dogs, monkeys, rats, and mice for pharmacologic information." This scheme is suggested in contrast to the "self-perpetuating choice," based on economic or convenience factors, of the rat and dog as test species.

Balazs (1976) discussed the rat and dog combination for testing chemicals. He favored the rat because large numbers can be used, and it has a relatively short life span. The choice of the dog allows for "in-depth studies of organ systems" and provides greater test sensitivity. The use of a third species is variable but "the omission of these species [rat and dog] is seldom justified." This policy is also recommended by the Food Safety Council (1978), and in the *EPA Proposed Guidelines for Pesticides* (*Federal Register*, 1978). The EPA position is stated as: "Testing shall be performed in at least two mammalian species. One species shall be a generally recognized strain of laboratory rat. The second species shall be a nonrodent. The nonrodent species should usually be the dog. Selection of a nonrodent species other than the dog will require full and adequate justification which should consider such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies." Thus, it is assumed that for most substances the combination of the dog and rat is preferred, although the scientific basis of this conclusion is not stated.

However, in contrast to many discussions concerning the dog and rat as test species, Aviado (1978) saw little advantage to including the dog. In a two-part literature review, Aviado evaluated 110 studies in which the dog and rat were used as test species during the period 1966 to 1978. In most studies the data obtained from the rat predicted cardiovascular, bronchopulmonary, hepatorenal, digestive, nervous, and somatic system toxicities as well as did data obtained from the dog. Further, the rat studies were better than the dog studies for prediction of pancreatic hypertrophy, hepatic porphyria, and nephropathy. He concluded that the use of the dog or the rat in chronic studies is sufficient and that using both species results in unnecessary duplication of scientific manpower and research effort.

The review by the World Health Organization (1978) on toxicity evaluations summarizes most of the primary factors discussed in literature reviews for the selection of test species. Of paramount importance is the similarity of metabolism between the test species and man. If sufficient data are available to allow a choice on this basis, the species showing the greatest metabolic similarity to man should be used. If such data are unavailable, then qualitative and quantitative response patterns should be obtained for several test species to increase the predictive value for the occurrence of similar toxic effects in man. In most cases this necessitates the use of the dog and rat as a minimum in subchronic studies. However, despite such reviews as the World Health Organization document and Aviado's literature review, the conclusion of Fancher (1978) in his review of animal models for toxicological studies is in essence the current general practice in species selection: "In spite of all that has been done, choice of species continues to be based, in large part, on precedence, convenience, and economics."

4.2.3 Species Comparison Studies

Ansbacher, Corwin, and Thomas (1942) used the dog, cat, rabbit, and monkey to test menadione and menadiol for toxicity. After subcutaneous dosing for 4 to 11 days, all species except the monkey developed a weak anemia, with the dog also showing hemoglobinuria. The dog and cat showed some mortality at the higher doses. In the discussion of the results, Ansbacher et al. felt that the hematologic changes could be attributed to normal compensatory mechanisms. Thus, the mortality exhibited by the cat and dog implies a greater sensitivity for these two species than for the rabbit and monkey.

In an inhalation study of beryllium toxicity, Stokinger et al. (1950) used 11 species, examining mortality, growth retardation, hematology, and urinalysis factors. The animals were exposed 6 h per day for 14 days at 100 mg/m^3, 51 days at 47 mg/m^3, 95 days at 10 mg/m^3, and 100 days at 0.95 mg/m^3. For mortality the rat and cat were the most sensitive species at intermediate and high doses, with the dog showing equal mortality rates at the high dose only. In growth response, the monkey was the most affected species at the intermediate and high doses.

The dog showed the most toxic effects in hematologic and urine analyses In summation, the dog appeared to be the most sensitive species, but the use of only five animals per test dose may limit the validity of this study. Stokinger and Stroud (1951) tested the dog, rat, and rabbit once again for hematological effects from beryllium inhalation. The exposures were 6 h per day, 5 days a week for 6 weeks (continued for 23 weeks in the rabbit) at 2.2 to 4.0 mg/m^3. The result was a decrease in red blood cells and an increase in mean corpuscle volume. Their comparison showed that the degree of anemia varied among species, with the dog being the most susceptible and the cat the least susceptible.

Rowe et al. (1952) compared the sensitivity of the rat, guinea pig, rabbit, and monkey to ethylene dibromide using repeated 7-h per day inhalation exposures with concentrations of 100, 50, and 25 ppm. Subchronic exposures of less than 91 days showed toxic effects only at the higher doses. All species showed some weight loss, but only changes in the guinea pig were significant. Mortality levels were statistically significant at the high dose in rats, as were increases in liver and kidney weights. This increase in organ weight was also shown by the guinea pig. Although Rowe et al. concluded that no species did particularly well at high dose, it is apparent that the rat and guinea pig showed the greatest sensitivity to ethylene dibromide.

Litchfield (1961) designed a study to compare experimental animal response and human response to toxic chemicals. By comparing the occurrence of toxic effects from tests performed on the rat and dog, and from known human epidemiological studies, Litchfield was able to evaluate the relationships for six chemicals. The tests included acute studies, subchronic studies (1 to 6 months), and a 1-year rat study. The appearance of toxic effects in each species and in each species combination was the basis for his evaluation. The results are shown in Table 4.3. The data support his conclusion that the dog better represented man's response than did the rat. However, in summation, Litchfield noted that the small number of chemicals examined reduced the statistical value of this comparison, and he urged that more species be examined for use in toxicity studies.

Table 4.3. Occurrence of 39 physical signs from six drugs in three species

Physical sign	Absent	Rat	Dog	Rat and dog	Man	Rat and man	Dog and man	Rat, dog, and man
Weight loss				2				4
Weight gain	3	1			2			
Muscle atrophy	4				1			1
Myositis	5						1	
Lymphocytopenia	4		1					1
Neutropenia	5			1				
Leukopenia	3		1		1			1
Anemia	1		2		1			2
Leukocytosis	3		1		2			
Hyperglycemia	5							1
Liver damage	2	1	2					1
Jaundice	5						1	
Fatty liver	5	1						
Polydipsia	5		1					
Polyuria	2				3		1	
Oliguria	4				1		1	
Hematuria	3		1				2	
Crystalluria or renal concretions	5					1		
Renal damage	3	1	1					1
Gastroduodenal ulcer	4		1				1	
Diarrhea	1		1		4			
Salivation	5		1					
Ataxia	2		1	1	1			1
Impaired reflexes	3			1	1			1
Decreased activity	3			2				1
Tremors	3				2		1	
Ptosis	5	1						
Catatonia	5	1						
Priapism	5	1						
Lacrimation	5		1					
Urinary incontinence	4	1			1			
Bacterial invasion	5						1	
Parasitic invasion	4				1		1	
Decreased thyroid function	4			1				1
Genital hypoplasia	4	2						
Decreased adrenal function (cortical)	5							1
Hypotension	2	1			2			1
Lung edema	5						1	
Tachypnea	5		1					
Total	146	11	16	8	23	1	12	17

Source: Adapted from Litchfield, 1961. Copyright 1961, American Medical Association. Reprinted with permission of the publisher.

In a similar study of anticancer drug toxicities, Owens (1962) compared the response of the rat, dog, and monkey to man in terms of bone marrow, gastrointestinal, hepatic, renal, and neural toxicities. Table 4.4 summarizes the results of 4-week exposure tests. Owens concluded that except for neurotoxicity all three species were suitable, and in particular he saw no advantage in the use of the monkey rather than the dog.

Weil and McCollister (1963) evaluated several factors in toxicity tests, including species sensitivity. Although their study concentrated on diet exposure studies in the rat, they also included some data on dog

Table 4.4. Organ system toxicities

Compound	Rodent	Dog	Monkey	Human
Bone marrow toxicity				
Mechlorethamine	+	+	+	+
Cyclophosphamide	+	+	+	+
Myleran	+	+	+	+
1-Sarcolysin	+	+	+	+
2-Chloroethylmethanesulfonate		+	+	?
Methotrexate	+	+	+	+
6-Mercaptopurine	+	+		+
4-Aminopyrazolo (3,4-D)pyrimidine	+	+		+
Vincaleukoblastine	+	+		+
Carzolamide	−	−		+[a]
Actinomycin P2	±	±		+[a]
Mithramycin	−	−		+[a]
Roseolic acid	−	+		+[a]
Gastrointestinal toxicity				
Mechlorethamine	+	+	+	+
Cyclophosphamide	+	+	+	+
Myleran	+	+	+	+
1-Sarcolysin	+	+	+	+
2-Chloroethylmethanesulfonate		+	+	+
Methotrexate	+	+	+	+
6-Mercaptopurine	+	+		+
4-Aminopyrazolo (3,4-D)pyrimidine	+	+		+
Carzolamide	−	+		+
6-Azauracil		±	−	+
Vincaleukoblastine	+	+		+
Actinomycin P2	+	+		+
Mithramycin	−	−		+

Table 4.4 (continued)

Compound	Rodent	Dog	Monkey	Human
Nervous system toxicity				
Mechlorethamine	+	+	+	+
Chloroquine mustard		+	+	+
Carzolamide	–	–		+
6-Azauracil	±	–	–	+
Vincaleukoblastine	–	–		+[b]
Nitrofurazone	–	–		+[b]
NSC 38280	–	–		+[c]
Skin and appendages toxicity (dermatitis and alopecia)				
Cyclophosphamide	–	–	–	+
Methotrexate	–	–		+
Vincaleukoblastine	–	–		+
8-Azaguanine	–	–		+
Actinomycin P2	–	–		+
Methylglyoxal-bis-guanylhydrazone	–	–		+
Hepatic toxicity				
2-Chloroethylmethanesulfonate	+	+		+
6-Mercaptopurine	+	+		+
4-Aminopyrazolo (3,4-D)pyrimidine	+	+		+
Carzolamide	±	+		+
Mithramycin	±	+		+
Roseolic acid	–	+		+
Renal toxicity				
2-Chloroethylmethanesulfonate	+	+		+
Aminoiminomethanesulfinic acid	+	+		+
Puromycin nucleoside	+	+		+

[a] Predominantly thrombocytopenia.

[b] Peripheral neuropathy.

[c] Extraocular palsies.

Source: Adapted from Owens, 1962. Reprinted with permission of the publisher.

studies, for durations up to 2 years. They found 21 studies in which the rat and dog were tested together. In none of these tests was the dog more sensitive, and in seven tests it was less sensitive than the rat. They concluded that the use of the dog should be limited to 90-day studies, and only when it was indicated as a more sensitive species should it replace or augment the rat in long-term studies.

The use of the pig as a test species was discussed by Earl et al. (1964). By injecting 1, 2, and 4 mg/kg of amphotericin B intravenously for 3 days per week over 13 weeks, they were able to test the similarity of the pig's response to that of man. Unlike previous rat and dog studies on amphotericin B, the toxic effects appearing in the pig (renal damage and anemia) were the same as human responses. They concluded that the pig may be a useful test species because of these similarities to man and because of the ease with which blood and organ samples are taken.

In a study of the pesticide zenophos, Kohn, Kay, and Calandra (1965) orally dosed the rat and dog for 90 days with diet levels of 0.5, 2, 8, and 25 ppm. At the high and intermediate doses both species exhibited lowered food consumption, depressed growth rates, and depressed cholinesterase activity. Additionally, the dog showed changes in its hematologic pattern. The effects shown were attributed (except for cholinesterase activity) to poor diet palatability and not to species differences.

McNerney and MacEwen (1965) studied the inhalation toxicities of ozone, carbon tetrachloride, and nitrous oxide in five test species after continuous exposure for 2 weeks. The effects were generally the same for all five species, except for the greater susceptibility to nitrous oxide and a resistance to ozone exhibited by the monkey. In contrast, the dog showed the opposite pattern of effects. Also, a strain difference in the rats appeared significant, as Wistar rats were extremely susceptible to carbon tetrachloride while Sprague-Dawley rats were not.

A study using the rat and dog to test the oral toxicity of EX5004, a sympatholytic drug, showed that after a 3-month exposure the dog was more affected than the rat (Yeary, Brahm, and Miller, 1965). The exposure levels were 10, 100, 500, or 1000 mg/kg in the diet for the rat and for the dog 10, 100, or 500 mg/kg given by gelatin capsule. The dog showed tachycardia and elevation of alkaline phosphatase activity at the high and intermediate doses while the rat did not. This response was closer to the expected human response pattern, indicating that for these variables the dog was the most sensitive test species.

Atkinson et al. (1966) studied the effects of cephaloridine, an antibiotic, by using daily intramuscular or subcutaneous injections into four species (cat, dog, rat, and rabbit) for 56 to 84 days. The major significant effect was an increase in kidney weight in the cat (at 50 and 150 mg/kg), rabbit (at 30 and 45 mg/kg), and rat (at 50 and 150 mg/kg). However, the rat was the only species showing kidney damage. There was also a slight, transient leukocytopenia in the rat and dog and some enlargement of the liver (without histopathologic damage) in the rabbit and cat. In this study it appeared that the rat would have been sufficient as a test species.

Newberne, Gibson, and Newberne (1967) studied an analgesic drug in the dog, rat, and monkey using oral dosing for 1 to 4 months. As shown in Table 4.5, the dog was overwhelmingly more sensitive than the rat or monkey, especially for histopathologic and clinical chemistry effects. The only significant effect that appeared in the monkey and not in the dog was the demyelination of the cerebral gyri. Thus, overall the dog was the most sensitive species in this study.

Hagan et al. (1967) compared the toxicities of food flavorings using oral exposure by diet or gavage for the dog (beagle) and the rat (Osborne-Mendel). For 6-methylcoumarin, they found at the intermediate dose (10 000 ppm for rat and 150 mg/kg for dog) that 20 rats showed no effect after 14 weeks while the dog tested became moribund after 5 weeks, exhibiting liver damage, skeletal muscle damage, and general weakness. For methyl salicylate the rat showed growth retardation after 17 weeks at the intermediate dose (10 000 ppm). The dog showed weight loss and slight microscopic liver damage after no more than 29 days at 800 mg/kg. One dog died after 4 days of dosing. With safrole ingested at intermediate doses (5000 ppm), the rat exhibited growth retardation, liver pathology, slight leucocytosis, and anemia after dosing for 2 years. The dog, however, showed moderate kidney and liver damage and general weakness after 96 to 116 days at 40 mg/kg. In these studies, the dog generally appeared to be more sensitive to the additives than the rat, even with shorter exposure periods.

Table 4.5. Comparison of significant variations in the response of four species to an oral analgesic[a]

Parameter	Dog	Dosage[b] (mg/kg/day)	Monkey	Dosage[b] (mg/kg/day)	Rat	Dosage[b] (mg/kg/day)	Rabbit	Dosage[b] (mg/kg/day)
Clinical observations								
Depressed weight gain or weight loss	+[c]	30	+	25	+	20	–[d]	80
Depression or unconsciousness	+	30	+	30	–	100	–	80
Emesis	+	30	+	25	–	100	–	80
Icterus	+	30	–	60	–	100	–	80
Death	+	30	+	30	–	100	–	80
Clinical chemistry								
Elevated serum glutamic pyruvic transaminase	+	10	–	60	–	100	NT	NT
Elevated serum alkaline phosphatase	+	10	–	60	–	100	NT	NT
Bilirubinuria	+	60	–	60	NT[e]	NT	NT	NT
Necropsy observations								
Discolored, yellow liver	+	30	–	60	–	100	–	80
Histopathologic observations								
Hepatic necrosis	+	30	–	60	–	100	NT	NT
Myelin figures in hepatocytes	+	30	+	30	–	100	NT	NT
Excess bilirubin – liver and kidney	+	30	–	60	–	100	NT	NT
Hemosiderosis	+	30	–	60	–	100	NT	NT
Depressed spermatogenesis	+	30	–	60	–	100	NT	NT
Demyelination of cerebral gyri	–	60	+	30	–	100	NT	NT

[a] 1-2-(1-Methyl-2-piperidyl)-1,1-diphenylethyl propionate hydrochloride.

[b] Minimum dose at which an effect occurred or the highest dose given.

[c] Indicates a response in one or more animals at the indicated dose.

[d] Indicates no abnormal response.

[e] No test.

Source: Adapted from Newberne, Gibson, and Newberne, 1967. Reprinted with permission of the publisher.

In a toxicological study of aldrin and dieldrin, Hodge, Boyce, Deichmanne, and Kraybill (1967) noted the subchronic response of several species by comparing histopathologic data, body weight changes, mortality, and organ weight changes. Using these criteria, they found wide variability in species response. They found the monkey was the most sensitive for body weight and mortality criteria, and both the dog and rat were sensitive to histopathology and liver weight criteria. In this study it was the sensitivity of the species that was evaluated and not the similarity to human responses. The significance of this study lies in the selection of the rat, dog, and monkey as the best indicators of toxicity from among a list of ten species.

Benitz, Roberts, and Yusa (1967) studied the morphological changes in the rat, dog, monkey, and mouse after dosing for 27 to 38 days with minocycline, a tetracycline antibiotic. In the rat, minocycline produced a black discoloration of the thyroid (pigment deposition) with oral doses of 8, 25, and 75 mg/kg per day. Hyperplastic changes were associated with this discoloration. In the dog, the discoloration and hyperplasia occurred with intravenous doses of 5, 10, 20, and 40 mg/kg per day. Additionally, various degrees of hemolytic anemia occurred in the dose range 10 to 40 mg/kg per day. In the monkey, the discoloration occurred (pigmentation less pronounced) at an oral level of 30 mg/kg per day, but no hyperplastic changes were observed. However, in the mouse, no changes of any kind were noted with 250 mg/kg per day of oral dosing. The results in this limited evaluation of toxicity indicated that the rat and dog were the most sensitive species, followed by the monkey. The mouse was the least sensitive species tested.

Schein et al. (1970) evaluated the monkey (four species) and dog (mongrel and beagle) for their potential value in predicting qualitative toxicities in man using 25 anticancer drugs. They used data from their own tests (3 to 90 days of intravenous or peroral administration), data found in the literature, and data from clinical studies in man. They evaluated approximately 170 parameters to determine toxicities and, as shown in Table 4.6, found good predictive value by both animals for most organ systems. The dog was generally a better predictor for the occurrence of a toxic effect in man, while the monkey was better at predicting

Table 4.6. Predictive abilities of the dog, monkey, and combination

Organ system	TP[a] (%)	FP[b] (%)	TN[c] (%)	FN[d] (%)	$\frac{\text{FNS}[e]}{\text{TP + FN}}$ (%)	Number of compounds
Dog as a predictor for organ-specific toxicity in man						
Injection site	16	36	40	8	33	25
Integument	12	32	40	16	57	25
Cardiovascular	28	24	36	12	30	25
Respiratory	16	64	16	4	20	25
Bone marrow	80	12	0	8	9	25
Lymphoid	4	72	24	0	0	25
Gastrointestinal	92	8	0	0	0	25
Liver	52	44	4	0	0	25
Renal	32	56	4	8	20	25
Neuromuscular	24	60	12	4	14	25
Monkey as a predictor for organ-specific toxicity in man						
Injection site	13	26	52	9	40	23
Integument	13	17	57	13	50	23
Cardiovascular	22	26	30	22	50	23
Respiratory	13	48	30	9	40	23
Bone marrow	83	13	0	4	5	23
Lymphoid	0	31	65	4	100	23
Gastrointestinal	74	9	0	17	19	23
Liver	52	35	13	0	0	23
Renal	35	48	13	4	11	23
Neuromuscular	22	30	39	9	28	23
The combination of dog and monkey as a predictor for organ-specific toxicity in man						
Injection site	16	36	40	8	33	25
Integument	24	36	36	4	14	25
Cardiovascular	36	32	28	4	10	25
Respiratory	16	76	4	4	20	25
Bone marrow	88	12	0	0	0	25
Lymphoid	4	76	20	0	0	25
Gastrointestinal	92	8	0	0	0	25
Liver	52	48	0	0	0	25
Renal	36	56	4	4	10	25
Neuromuscular	24	60	12	4	14	25

[a] True positive, toxicity was observed in both the animals and in man.

[b] False positive, toxicity was observed in the animals but not in man.

[c] True negative, no toxicity was observed in the animals and man.

[d] False negative, toxicity was not observed in the animals but was recorded in man.

[e] Corrected false negatives — an index of false negative prediction which analyzes for only those compounds which produced the specific toxicity in man.

Source: Adapted from Schein et al., 1970. Reprinted with permission of the publisher.

the absence of a toxic effect in man. This complementary pattern is demonstrated by the correct prediction of toxicity in nine out of the ten organ systems by the combination of data from the dog and monkey studies. However, this predictive ability was achieved only with a high percentage of false positives.

Worth et al. (1970) tested the antibiotic A204 by oral exposure for 90 days in the rat (0.06, 0.025, 1.0 mg/kg per day) and dog (0.5, 0.8, 1.25 mg/kg per day). They found no significant species differences measured by tests of hematology, clinical chemistry, or histopathology. However, the mortality rate was slightly greater for the rat, particularly at the intermediate dose, and so was the depression of the growth rate. Therefore, in this study, the rat appeared to be more sensitive than the dog.

Vogin et al. (1970) used the rat (T-DRL) and the dog (beagle) to study azotrek, a tetracycline phosphate complex-sulfamethizole formulation, in tests for oral toxicity with dosing for up to 33 weeks. The rat showed no effects at any dose level (125, 250, 500 mg/kg per day). However, the dog could not tolerate the high dose, exhibiting decreases in hematocrit and hemoglobin levels, increased thyroid weight, and one fatality. The dog appeared to be the most sensitive species in this study.

Lyon et al. (1970) tested acrolein for inhalation toxicity in the rat, dog, guinea pig, and monkey using repeated exposure studies (8 h per day, 5 days per week, for 6 weeks, with concentration levels of 0.7 and 3.7 ppm) and continuous exposure studies (24 h per day for 90 days, with concentrations of 0.22, 1.0, and 1.8 ppm). In the repeated exposure test, the only effect at the 0.7-ppm concentration was a chronic inflammation in the lungs of the dog and monkey. At the 3.7-ppm level, the effects included a significant decrease in weight gain for the rat, mortality in the monkey, and nonspecific inflammatory changes in the lung, liver, and kidney (most severe in the dog and monkey). The dog also developed bronchopneumonia. In the continuous exposure test at 0.22 ppm, the dog and monkey showed specific inflammatory changes in the lung, while the monkey, guinea pig, and dog had nonspecific inflammation in the liver, kidney, and heart. At 1.0 ppm, the dog and monkey showed ocular and nasal irritation and histopathologic damage to the trachea. Also, the rat suffered growth depression. At 1.8 ppm, the irritation and tracheal damage were again noticed only in the dog and monkey. The rat again showed a decrease in weight gain, and all species showed nonspecific inflammation in the lung, liver, and kidney. The authors concluded that the dog and monkey were the most sensitive species.

A comparison of the miniature pig and the dog (beagle) was made by Earl et al. (1971) using subchronic tests of 1 to 148 days for the dog and 1 to 69 days for the pig. The exposure levels were 5, 10, 25, 50, 100, 300, and 500 mg/kg per day for the pig and 5, 10, 25, 50, 100, 300, 400, and 500 mg/kg per day for the dog. Both species suffered mortality, with the pig first affected at 5-mg/kg levels while the dog was not affected until the 25-mg/kg level. Neither species showed a change in hematological values, but the dog did exhibit erratic increases at the high dose level in alkaline phosphatase, serum glutamic oxaloacetic transaminase, lactate dehydrogenase, ornithine carbamyl transferase, and amylase values. Histopathological examination revealed lesions of the small intestine in the dog, and the pig had hemorrhaging of the heart and fat deposits in the pancreas, in addition to the intestinal lesions. These results indicated that the two species reacted comparably to the pesticide diazon. The dog showed greater biochemical sensitivity; the pig was more sensitive to lower doses and showed more lesion damage.

Knapp, Busey, and Kundzins (1971) compared the sensitivity of the rat and dog to monochlorobenzene (MCB) for 93 or 99 days of oral dosing. The dog was more sensitive to MCB, showing mortality, increases in the activity of alkaline phosphatase and serum glutamic pyruvic transaminase, increased numbers of immature leucocytes, and gross or microscopic pathology for the liver, kidney, gastrointestinal mucosa, and hematopoietic tissues. The majority of the effects were at the high dose (272.5 mg/kg per day). The rat exhibited some growth depression and increased liver and kidney weights at the high (250 mg/kg per day) and intermediate (50.0 mg/kg per day) doses. The effects found in the rat were not as severe as those found in the dog.

Jones, Strickland, and Siegel (1972) used the rat (Sprague-Dawley), guinea pig (Hartley), monkey (squirrel), and dog (beagle) to test the inhalation toxicity of propylene glycol 1,2-dinitrate for 90 days with daily exposure levels of 67, 108, and 236 mg/m^3. The effects detected were similar among all four species and included liver pathology and changes in hematological-biochemical parameters. The appearance of fatty deposits in the liver first occurred at the low dose for the guinea pig and rat, but not until the intermediate-dose level for the dog and monkey. Increased methemoglobin levels were found at the high dose for all species but were more severe for the dog and monkey. The dog also showed decreased hemoglobin and hematocrit levels at the high dose. The monkey had decreased alkaline phosphatase and increased blood urea nitrogen levels at the intermediate and high doses, and some mortality at the high dose. The dog and monkey appeared to be the more sensitive species for effects in hematolcgical and biochemical parameters, and the rat was more sensitive for liver and kidney changes.

Verschuuren, Kroes, and Tonkelaar (1973) compared variability in species response to oral doses of an acaricide, tetrasul, over a 90-day test period using diet levels of 50, 200, 1000, and 3000 ppm. Growth retardation was evident for all species at the high dose, and for all species except the rat and mouse at the higher intermediate dose. Increased liver weights were found at the lower intermediate dose for all species except the rat, and for all species at higher doses. Histopathological damage of the liver was found in all species at and above the lowest dose. In conclusion, Verschuuren and his associates ranked the six species based on nine criteria with a rating of 1 to 6 for each criteria. Based on this system, five species were in a range of ±2.5 points, with the mouse separated from this group by +10 points. In the narrower context of the evaluation of chlorinated hydrocarbons, the sensitivity ranks were as follows: the miniature pig and rat were the most sensitive; the rabbit, guinea pig, and chicken were next; and the mouse was the least sensitive.

King, Shefner, and Bates (1973) studied the effects of chlorinated dibenzodioxins in the rat and mouse after oral dosing in the diet for 42 weeks. As shown in Table 4.7, the rat generally suffered higher mortality rates and more liver or lung damage than the mouse. Using these criteria, the rat was a more sensitive species than the mouse.

Table 4.7. Summary of dioxin oral administration data

Compound	Sex	Rat (35 per group)				Mouse (50 per group)			
		Week of test	Number of Survivors	Significant pathology		Week of test	Number of Survivors	Significant pathology	
				Lung	Liver			Lung	Liver
Controls	M	34	35			31	50		
	F	34	35			31	50		
1% Dioxane	M	42	24	8/11	1/11	40	50		
	F	42	20	3/5	2/5	43	49		
0.5% Dioxane	M	42	26	6/6	1/6	40	49		
	F	42	32	3/3	1/3	43	49		
1% Unsubstituted dibenzodioxin	M	42	33	2/2	2/2	39	48		
	F	42	32	1/2	2/2	39	29	3/14	7/14
0.5% Unsubstituted dibenzodioxin	M	42	31	3/4	1/4	34	50		
	F	42	35			39	49		
1% Dichloro-dibenzodioxin	M	17	35			17	49	0/1	0/1
	F	17	35			17	48	1/2	1/2
0.5% Dichloro-dibenzodioxin	M	17	35			17	50		
	F	17	35			17	49		
1% Octachloro-dibenzodioxin	M	32	0	1/5	5/5	10	0	0/5	5/5
	F	22	0	0/5	5/5	37	5	0/5	6/6
0.5% Octachloro-dibenzodioxin	M	37	0	1/5	5/5	8	0	0/5	5/5
	F	25	0			37	45		
0.25% Octachloro-dibenzodioxin	M	17	28	1/2	2/2	17	1		
	F	17	15	0/3	3/3	15	0	2/2	1/2
0.125% Octachloro-dibenzodioxin	M					9	0		
	F	17	30						

Source: Adapted from King, Shefner, and Bates, 1973.

Harris et al. (1973) compared the guinea pig (Hartley), rat (C-D), and mouse (albino CD-1) for sensitivity to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). They exposed each species to TCDD in the diet for 30 consecutive days and/or once a week for 4 to 8 weeks. As shown in Table 4.8 the guinea pig appeared to be the most sensitive species, followed by the rat.

Kast et al. (1975*a*) administered fentoterol-HBr to the mouse (ICR-JCL) and rat (Sprague Dawley-JCL) by gavage daily for 30 days. At the highest dose (1500 mg/kg), the rat showed growth depression; a high mortality rate; increased weight of the salivary glands, heart, and liver; and histopathological effects in the heart and salivary glands. At the highest dose (150 mg/kg), the mouse showed all the above effects except growth depression, liver weight increase, and heart histopathology. However, the mice did exhibit histopathological damage to the liver. Both species showed dose-dependent changes in hematology and biochemical factors. For the rat, this included increased blood urea nitrogen level, glutamic oxaloacetic transaminase activity, and glutamic pyruvic transaminase activity and decreased platelet and glucose levels. In the mouse, hemoglobin, red blood cells, and packed cell volume all increased in a dose-dependent manner. The rat appeared to be slightly more sensitive to fentoterol-HBr than the mouse.

In another study by Kast et al. (1975*b*), the toxicity of fominoben-HCl was studied by dosing the mouse (ICR-JCL) and the rat (Sprague Dawley-JCL) for one month by gavage. The daily exposure levels were 250, 500, 1000, and 2000 mg/kg for the rat and 500, 1000, 2000, and 4000 mg/kg for the mouse. Mortality occurred only at the high dose for both species. Pathological findings were limited to a significant dose-dependent increase in liver weight. At the highest dose, this increase occurred with a fatty degeneration in the mouse liver and with a focal necrosis followed by a widening of interstitial connective tissue in the rat liver. An increase of enzyme activity paralleled the lesion formations. In the rat, the enzymes and biochemical parameters affected included bilirubin, cholesterol, alkaline phosphatase, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase. The parameters affected in the mouse were not listed.

Table 4.8. Summary of biological effects of TCDD[a]

Frequency of dose and criteria affected	Rat			Guinea pig			Mouse
	Dose (μg/kg)	MTD[b] (days)	Mortality[c]	Dose (μg/kg)	MTD[b] (days)	Mortality[c]	Dose (μg/kg)
Lethal dose							
Single	100	18	6/14	3	18	9/10	>50
Weekly	4 × 25	28	2/10	5 × 1	28	10/10	>4 × 25
Daily	10	22	15/16				
Body weight							
Lowest dose effect							
Single	25			1			
Weekly	6 × 5			8 × 0.2			4 × 25
Daily	30 × 1						
No effect							
Single	5						50
Weekly	6 × 1			8 × 0.04			4 × 5
Daily	30 × 0.1						
Thymus weight							
Lowest dose effect							
Single	5						10
Weekly	6 × 5			8 × 0.04			4 × 5
Daily	30 × 0.1						

[a] 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin.

[b] Mean time to death after first exposure.

[c] Number of animals dying per number of animals treated.

Source: Adapted from Harris et al., 1973.

Villeneuve and Newsome (1975) tested hexachlorobenzene (HCB) in the rat (Wistar) and guinea pig by administering a daily peroral dose of 500 mg/kg body weight for 16 days. Both species showed high mortality, but the guinea pig was affected most. The rat showed high tissue concentrations indicating a greater resistance to HCB. Both species experienced weight loss before death. No histopathology, hematology, or biochemical tests were performed. On the basis of a greater mortality rate and lower tissue concentrations, the guinea pig appeared to be the more sensitive species to HCB.

The rat (Charles-River) and dog (beagle) were used to test TR2379, an antihypertensive agent, for peroral toxicity over a 13-week period (Hartnagel et al., 1975). For the rat the dose levels were 50, 200, and 820 mg/kg per day, and the dog was dosed at 30, 70, and 160 mg/kg per day. Growth retardation occurred for male rats at the intermediate and high doses and for female dogs at the high dose. Increased kidney, adrenal, and thyroid weights were recorded for male rats at the high dose, and while both sexes had increased liver and heart weights. In the dog only males had organ weight increases, and these were limited to the liver and kidney at all doses. Both species suffered mortality at the high dose. The sensitivity of the two species was about equal, with male dogs showing organ weight increases at low doses while male rats showed more growth depression at lower doses.

Koeferl et al. (1976) reported the toxicity of cyclohexanone to the rat, dog, and monkey after intravenous injection of 142 and 284 mg/kg per day 5 days per week for 21 days. The rat exhibited the highest mortality rate at an additional high dose (568 mg/kg). However, the dog showed erythroid hyperplasia with increased hematocrit counts, a myeloid-erythroid ratio below unity, and nucleated red blood cells in the peripheral blood. This was termed the "most significant" finding, indicating the authors' opinion concerning the value of the dog in this toxicity evaluation.

In a study of the antibiotic sisomicin, Robbins and Tettenborn (1976) tested intramuscular toxicity in the rat (Wistar and Sprague-Dawley) and dog (beagle) for 3 to 5 weeks and 13 weeks. For the shorter duration the daily dose levels were 2 to 100 mg/kg for the rat and 1 to

60 mg/kg for the dog. During the longer duration, the daily levels were 2, 4, and 8 mg/kg (rat) and 2 to 16 mg/kg (dog). At the higher doses, the rat experienced a 33% mortality rate, glycosuria, reduced body weight, increased blood urea nitrogen and serum creatinine levels, and renal histopathological effects in the shorter test. The 13-week exposure showed a dose-dependent response for renal damage. In the 5-week dog studies, renal damage and mortality were the primary effects, and this trend continued for the 13-week exposure. The renal pathology was again dose dependent and occurred to a greater degree at lower doses in the dog than in the rat. The wider response spectrum shown by the rat offsets the greater sensitivity at low doses shown by the dog, so neither species showed a definite advantage in this study.

In a study of drug metabolism, Litterst et al. (1976) compared the monkey (squirrel), rat (Sprague-Dawley), miniature pig (Hanford), and common tree shrew (*Tupaia glis*) as replacements for the rhesus monkey. Each species was evaluated for its enzymatic activity pattern by an examination of 14 parameters of drug metabolism in hepatic microsomal and soluble fractions, including liver weight to body weight ratio, cytochrome P-450, glutathione *s*-aryltransferase activity, reduced nicotinamide-adenine dinucleotide phosphate-cytochrome C reductase activity, mixed-function oxidation, aminopyrine *N*-demethylase activity, ethylmorphine *N*-demethylase activity, biphenyl hydroxylase activity, aniline hydroxylase activity, aryl hydrocarbon hydroxylase activity, UDP-glucuronyltransferase activity using both *p*-nitrophenol and *o*-aminophenol acceptor substrates, and *N*-acetyltransferase activity using both *p*-aminobenzoic and sulfadiazine acceptor substrates. All four species demonstrated activity in 13 of the 14 parameters, but no one species was similar to the rhesus in all quantitative aspects. Based on an arbitrary scale of favorable response, the similarity to the rhesus is as follows: the miniature pig is closest, followed by the rat, the squirrel monkey, and the tree shrew. In conclusion, the authors felt that the miniature pig would be suitable if housing and handling problems could be solved and that in any case a primate replacement for the rhesus might not be the best choice.

Sulfolane, a manufacturing solvent, was tested for inhalation toxicity by repeated (8 h per day, 5 days per week, for 27 exposures) and continuous (23 h per day for 85 to 110 days) exposures of the rat (Sprague-Dawley), guinea pig (Hartley), monkey (squirrel), and dog (beagle) (Anderson et al., 1977). In the repeated exposure test a dose concentration of 495 mg/m^3 was used, and in the continuous exposure test the levels were 2.8, 4.0, 20, 159, and 200 mg/m^3. All animals except the monkey survived the repeated-dose test. Of nine monkeys exposed to the high dose, three died and five were sacrificed in a moribund state after 17 days. All had pale livers and hearts, and five showed fatty metamorphosis of the liver. No significant hematological, body weight, or biochemical changes were noted in the other species. All species showed chronic lung inflammation, with the rat also showing liver inflammation. In the continuous study, the monkey again exhibited high mortality rates at the high dose level. The dog, which was exposed to the high dose, exhibited highly aggressive behavior, seizures and pneumonia. No body weight changes were noted in the rat or the guinea pig although the histopathology examination revealed liver damage. The guinea pig also showed some leukopenia and increased plasma transaminase activity at the high dose. All species again showed chronically inflamed and hemorrhagic lung tissue at the two higher doses. In their discussion of the results, the authors rated the susceptibility of the species to sulfolane in a monkey-dog-rat progression.

Cavender et al. (1977) compared the sensitivity of the rat (male Charles River) and guinea pig (female Hartley) to sulfuric acid mist (5.0 and 10.0 mg/m^3), ozone (1.0 and 2.0 ppm), and a combination of both. Both species were exposed via inhalation for 2 and 7 days. The guinea pig, which proved to be more sensitive to sulfuric acid mist than the rat, exhibited lung damage at low doses and mortality at higher doses. With ozone, both species showed lung damage and increased liver weight to body weight ratios, but the rat adapted to this stress more quickly than did the guinea pig. In conclusion, the authors pointed out that the differences in sensitivity between the rat and the guinea pig illustrate the importance of using "at least two species in inhalation toxicity studies."

In a test of the inhalation toxicity of acrolein, a cigarette smoke component, Feron et al. (1978) exposed the rat (Wistar), rabbit (Dutch), and hamster (Syrian Golden) to the chemical for 6 h per day, 5 days per week, for 13 weeks. As shown in Table 4.9, all three species were affected. The rat was the most sensitive. It suffered mortality and showed toxic effects at the lowest dose, unlike the hamster and rabbit.

One aspect of species selection that has not been considered by other reports is the opposition of the general public to the use of some nonrodent species as test animals because of their status as domestic pets. As a result of such a consideration, Cummings et al. (1979)

Table 4.9. Summary of treatment-related effects in hamsters, rats, and rabbits repeatedly exposed to acrolein (ppm) for 13 weeks

Criteria affected	Hamster			Rat			Rabbit		
	0.4	1.4	4.9	0.4	1.4	4.9	0.4	1.4	4.9
Symptomatology	0[a]	x	xxx	0	x	xx	0	x	xxx
Mortality	0	0	0	0	0	+++	0	0	0
Growth	0	0	**	*	**	***	0	*	**
Food intake	NE[b]	NE	NE	0	*	**	0	*	**
Hematology	0	0	x	0	0	0	0	0	0
Urinary amorphous material	0	0	+	0	0	+	0	0	+
Urinary crystals	0	0	*	0	0	*	0	0	0
Organ weights									
Lungs	0	0	++	0	0	++	0	0	++
Heart	0	0	+	0	0	+	0	0	0
Kidneys	0	0	+	0	0	+	0	0	0
Adrenals	0	0	0	0	0	+++	0	0	0
Gross pathology									
Lungs	0	0	0	0	0	x	0	0	0
Histopathology									
Nasal cavity	0	x	xxx	x	xx	xxx	0	0	xx
Larynx	0	0	x	0	0	xx	NE	NE	NE
Trachea	0	0	xx	0	0	xxx	0	0	x
Bronchi and lungs	0	0	0	0	0	xxx	0	0	xx

[a] 0 – Not affected; x – Slightly affected; xx – Moderately affected; xxx – severely affected; * – Slightly decreased; ** – Moderately decreased; *** – Markedly decreased; + – Slightly increased; ++ – Moderately increased; +++ – Markedly increased.

[b] Not examined.

Source: Adapted from Feron et al., 1978. Reprinted with permission of the publisher.

evaluated the rat as a replacement for "pet" species (the dog, cat, etc.). They specifically tested some physiological and behavioral techniques, primarily used in larger animals, and evaluated their potential as toxicity indicators in the rat. Included in this evaluation were techniques for detecting changes in blood pressure, heart rate, electrocardiogram (ECG) patterns, respiratory measurements, treadmill performance, various reflex and body regulatory mechanisms, body temperature, and behavior (retaining learned behavior, learning new behaviors, and maintaining unconditioned behaviors). Changes in these physiological and behavioral traits can indicate toxic effects in the respiratory, cardiovascular, neuromuscular, central nervous, and autonomic nervous systems. The techniques were all performed using restrained but unanesthesized rats to avoid interfering effects from the anesthetic. The results indicated that the rat could be analyzed for toxic effects using these techniques and would be a suitable replacement for larger test species.

4.2.4 Conclusions

In evaluating the suitability of species for toxicity tests, one constantly finds conflicting data. Depending upon the chemical used, the rat, dog, monkey, and guinea pig have each been selected in various studies as the most sensitive species. This trend indicates why there is such reluctance to change from the standard dog-rat combination. In papers discussing the value of rodents (mouse, guinea pig, rabbit, rat, and hamster), the guinea pig and rat usually prove to be the most sensitive. The studies by Kast et al. (1975*a*, 1975*b*), Benitz, Roberts, and Yusa (1967), and King, Snefner, and Bates (1973) eliminate the mouse as a potential test species for subchronic toxicity tests, while studies by Stokinger et al. (1950) and Feron et al. (1978) eliminate the other rodent species. In direct comparisons of the guinea pig and rat, evidence can be found to support either species as the most sensitive. In their studies, Jones, Strickland, and Siegel (1972) and Roe (1968) ranked the rat as more sensitive than the guinea pig, but Harris et al. (1973) and Villeneuve and Newsome (1975) found just the opposite.

Additionally, Rowe et al. (1952) and Cavander et al. (1977) all considered the sensitivity of both species to be equal. The selection of a preferred species in this case may have to be based on economic factors or on individual species response to certain classes of chemicals.

The value of including a nonrodent species in the test protocols is also a source of considerable contradiction and disagreement. Some authors feel that the use of the dog-rat-monkey combination should be encouraged (Anderson et al., 1977; Freireich et al., 1966; Guarino, 1979; Hayes, 1967*b*; Hodge, Boyce, Diechmanne, and Kraybill, 1967; Jones, Strickland, and Siegel, 1972; McNerney and MacEwen, 1965; Owens, 1962; Zbinden, 1963) at least for some chemicals. Others feel that the rat and dog are sufficient to detect most toxic effects (Balazs, 1976; Hartnagel et al., 1975; Kohn, Kay, and Calandra, 1965; Robbins and Tettenborn, 1976). If no information on the metabolism and pharmacokinetics of the chemical in question is available, the literature cited would support the recommendation that the studies be done using the dog and the rat. However, as discussed in Sect. 4.2.2, if data on metabolism in animals are available that can be related to man, this information should be of paramount importance in choice of species. Before embarking on lengthy, expensive animal studies, efforts should be made to ensure that the animals chosen for the studies are not unique in the way they metabolize the test chemical. The same effort should be made in regard to the physiological functional pattern of the test species, since differences in this area can be just as important as metabolic differences. In direct comparisons, the dog is usually more sensitive than other nonrodent species, such as the monkey or cat, although Smith (1979) did find the monkey to be metabolically more similar to man than was the dog. The dog has also been selected as the most sensitive species in more studies (Ansbacher, Corwin, and Thomas, 1942; Hagan et al., 1967; Knapp, Busey, and Kundzins, 1971; Koeferl et al., 1976; Litchfield, 1961; Newberne, Gibson, and Newberne, 1967; Stokinger et al., 1950; Stokinger and Stroud, 1951; Vogin et al., 1970; Yeary, Braham, and Miller, 1965) than the rat (Atkinson et al., 1966; Verschuuren, Kroes, and Tonkelaar, 1973; Weil and McCollister, 1963; Worth et al., 1970). The review by Aviado (1978) suggested that the use of both the dog and rat is superfluous and that the rat alone may be sufficient. This trend is also supported by the work of Cummings et al. (1979) in which physiological and behavioral assessment techniques, usually restricted to use with larger animals, were found to be applicable in rat studies. To further complicate the issue, Earl et al. (1964, 1971) gave good evidence supporting the use of the miniature pig in certain instances. When the scientific data are conflicting, economic and species availability factors should also be considered in the task of species selection.

It is obvious that, in the ideal situation of maximum detection of toxic effects, a dog-rat combination should be used with selected incorporation of alternative species such as the guinea pig, monkey, or miniature pig. The exact combination would depend on the chemical or effect to be evaluated. The analysis by Gehring, Rowe, and McCollister (1973) indicated that cost and time parameters could be very high for a subchronic test using two species. Therefore one could infer that the ideal situation would be to select a single test species to reduce these costs. However, if by using two species, such as the dog and rat, a more efficient determination of toxicity is achieved, then a savings in both time and cost could be managed by reducing or eliminating the need for costlier chronic or lifetime studies. This aspect of the problem will be discussed further in the section on subchronic test durations.

4.3 DURATION

4.3.1 Introduction

The duration of a subchronic test has always been a poorly defined parameter. As discussed in the introduction, even the definition of a subchronic test, which is based in large part on duration. has not specified a standard exposure duration. The closest to a standard exposure duration is 90 days. Therefore, for this discussion the 90-day subchronic test will be considered the standard duration. However, tests of shorter durations, especially 28- or 30-day tests, in the literature are also discussed. Durations of 14, 28, and 90 days are commonly used and guidelines indicate that these are acceptable. The 14- to 28-day tests are generally used to provide information on the nature of the toxic effect, and likely "no effect" and "effect" doses. They do not usually provide information on maturation or aging effects. The 90-day duration can provide information on the effects of chemicals on the maturation process, in addition to evaluating the nature of toxic effects at lower prolonged doses. The purpose of this section is to evaluate the effectiveness of 90-day and shorter duration tests by presenting duration reviews from the literature and summarizing data from duration comparison studies.

4.3.2 Duration Reviews

One of the first discussions of the length of exposure necessary for a repeated-dose study was that of Smith (1950). As a result of the need for antimalarial drugs during World War II, a short test of 11 to 14 days was designed (Wiselogle, 1946) to rapidly evaluate the toxicity of these drugs. Smith later evaluated the effectiveness of this test, including increasing the duration to 21 days, by comparing growth rate curves and particularly the size of the standard deviations. He assigned an arbitrary value of 100% for the sensitivity of the method at 21 days and calculated what percent of this sensitivity was achieved at shorter durations. The results were 15% to 30% sensitivity at 7 days, 68% to 85% sensitivity at 11 days, and 75% to 95% sensitivity in 14 days. He concluded that for absolute toxicity evaluations the gain was significant when the test was increased from 11 to 14 days but not when increased to 21 days. Thus, Smith recommended a subchronic test duration of 14 days.

In a discussion of the relationship between short- and long-term oral toxicity studies, Weil and McCollister (1963) evaluated the effectiveness of a 90-day diet test. Their evaluation was based on 33 chemicals using the criteria of body weight gain, relative weight changes of the liver and kidney, and liver and kidney pathology studies in the rat. They compared both the minimum effect and maximum no-effect levels for short-term (approximately 90 days) and long-term (2-year) studies, arriving at a 90-day:2-year ratio for each level (Table 4.10). These ratios indicated the degree to which these values changed with increased test duration. In 95% of the studies the ratio was 6.0 or less, meaning that the safe levels found for a chemical tested for 90 days could be

Table 4.10. Relationship of dosage levels of short-term and 2-year feeding of materials in the diet of rats

Material number	Duration of short-term test	Percentage of materials in diet				Ratio of short-term to 2-year test	
		Short-term		2-years			
		Minimum effect	Maximum no-effect	Minimum effect	Maximum no-effect	Minimum effect	Maximum no-effect
1	105	0.015	0.005	0.03	0.01	0.5	0.5
2	90	4.0	2.0	8.0	4.0	0.5	0.5
3	90	1.0	0.3	2.0	0.2	0.5	1.5
4	120	3.0	1.0	5.0	0.5	0.6	2.0
5	90	0.25	0.0625	0.256	0.064	1.0	1.0
6	90	0.01	0.003	0.01	0.003	1.0	1.0
7	97	0.1	0.03	0.1	0.03	1.0	1.0
8	90	8.0	4.0	8.0	4.0	1.0	1.0
9	130	1.0	0.3	1.0	0.2	1.0	1.5
10	30	0.05	0.012	0.04	0.01	1.2	1.2
11	30	25.0	10.0	20.0	5.0	1.2	2.0
12	90	0.75	0.375	0.40	0.13	1.9	2.9
13	90	10.0	3.0	5.0	1.0	2.0	3.0
14	90	0.03	0.01	0.0125	0.0062	2.4	1.6
15	130	3.0	1.0	1.0	0.2	3.0	5.0
16	50	0.3	0.1	0.1	0.03	3.0	3.3
17	98	0.01	0.003	0.003	0.001	3.3	3.0
18	90	16.0	8.0	4.0	2.0	4.0	4.0
19	29	0.25	0.06	0.06	0.02	4.2	3.0
20	210	0.25	0.05	0.05	0.01	5.0	5.0
21	90	0.225	0.15	0.04	0.02	5.6	7.5
22	130	0.1	0.03	0.005	0.0025	20.0	12.0
23	90	0.5	0.25	M[a]	0.5		0.5[b]
24	90	0.009	0.003	M[a]	0.004		0.8[b]
25	30	0.[illegible]	0.1	M[a]	0.1		1.0[b]
26	90	8.0	4.0	M[a]	2.0		2.0[b]
27	90	16.0	8.0	M[a]	4.0		2.0[b]
28	90	M[a]	3.0	3.0	1.0		3.0[c]
29	93	M[a]	5.0	5.0	1.0		5.0[c]
30	91	M[a]	0.18	0.06	0.02		9.0[c]
31	90	M[a]	1.0	M[a]	0.3		3.3[d]
32	90	M[a]	2.5	M[a]	0.5		5.0[d]
33	142	M[a]	25.0	M[a]	5.0		5.0[d]

[a] Indicates that the maximum no-effect level was the highest dosage level fed.

[b] As the M level was on the 2-year test, the ratios are a maximum.

[c] As the M level was on the short-term test, the ratios are a minimum.

[d] As the M levels were on both the short-term and 2-year tests, the ratios are indicative only of which levels were used.

Source: Adapted from Weil and McCollister, 1963. Reprinted with permission of the *Journal of Agricultural and Food Chemistry*. Copyright by the American Chemical Society.

used to predict results for a 2-year study, with a significant degree of accuracy, by dividing the short-term value by 6.0. Since it is common to apply a hundredfold decrease, as a safety factor, to the maximum no-effect level determined with a 2-year study, the sixfold difference resulting from the 90-day study would appear to be a reasonable estimate of chronic effects. However, Weil and McCollister did not recommend that the 2-year study be abandoned, stating only that in certain cases a

90-day study would be sufficient. The determination of when the shorter study would suffice is left up to the toxicologist based on his experience with the chemical and its metabolic pattern.

In a later paper, Weil et al. (1969) examined the 90-day test in the same manner to see if a shorter test could predict the results it gives. Their evaluation included the prediction of 90-day repeated exposure results from 7-day repeated exposure and acute LD_{50} data. They tested the oral toxicity of 20 chemicals in the diet of the rat using acute LD_{50}, 7-day durations, and 90-day durations and evaluating the same criteria used by Weil and McCollister in 1963. By constructing minimum effect level ratios between each duration group (acute, 7-, and 90-day) as they had done with 90-day:2-year minimum effect levels, Weil et al. (1969) were able to evaluate the predictive relationships (Table 4.11). The relationship between the LD_{50} data and the 90-day test was poor, needing a ratio of 40 to achieve a predictive accuracy of 95%. The relationship between LD_{50} data and 7-day test data was not much better, with a ratio of 20 for 95%. However, extrapolation from 7- to 90-day tests was much better, with a ratio of only 6.2 for 95% of the results.

Table 4.11. Parameters of ratios of acute peroral LD_{50}s, 7- or 90-day and 2-year minimum effect (MiE) dosage levels

Percentile	Formula or symbol	Ratio			
		LD_{50} to 7-day MiE	LD_{50} to 90-day MiE	7-day MiE to 90-day MiE	90-day MiE to 2-year MiE
25th	$Q_1 = P_{25}$	1.2	2.3	2.2	1.1
50th	P_{50} = median	2.3	10.0	3.0	1.8
75th	$Q_3 = P_{75}$	6.0	25.0	5.2	3.8
95th	P_{95}	20.0	40.0	6.2	5.7
Semiinterquartile range	$(Q_3 - Q_1)/2$	2.4	11.4	1.5	1.4
Coefficient of rank correlation	P	0.831	0.782	0.943	0.946

Source: Adapted from Weil et al., 1969. Reprinted with permission of the publisher.

This compares favorably with the extrapolation results of the 2-year studies done in 1963. As the authors stated: "Therefore, one can predict 90-day results from a 7-day test with the same confidence as one can the two-year results from a 90-day test." The formulas necessary to predict the minimum effect dose for 50% to 95% of the studies are given in Table 4.12. These two studies were among the first to present sufficient significant data indicating that subchronic test durations could be used to predict chronic effect levels without a substantial loss in sensitivity.

Table 4.12. Prediction formulas

Value	Predicted upper limit to achieve a minimum effect level (MiE)	
	For 90-day feeding study	For 2-year feeding study
Median	7-day MiE/3.0[a]	90-day MiE/1.8[a] or 7-day MiE/5.4
95th percentile	7-day MiE/6.2	90-day MiE/5.7 or 7-day MiE/35.3

[a] The denominators for the 7-day MiE/2-year MiE relationships were obtained by multiplying those for 7 and 90 days [e.g., 5.4 = 3.0 (1.8)].

Source: Adapted from Weil et al., 1969. Reprinted with permission of the publisher.

Peck (1968) discussed the value of shorter tests in drug evaluation studies. He cited a World Health Organization Technical Report (1966) and Davey (1964) as recommending that a 3- or 6-month test is adequate to detect long-term toxicity. This 3- to 6-month duration is also recommended by Boyd (1968), Bein (1963), Barnes and Denz (1954), and Zbinden (1963). Peck gave data from studies done from the previous 15 years to support the use of a duration of 3 months. As shown in Table 4.13, only one study showed additional toxicity after 3 months, and only four showed added toxicity after 2 months. This again supports

Table 4.13. Approximate duration of drug administration required to define toxicity[a] in animals

Compound	Duration (months)							
	1/2	1	2	3	6	12	18	24
Indomethacin	x[b]	x	0[c]	0	0	0	0	0
Hydrochlorothiazide	x	x	0	0	0			
Amiloride HCl	x	x	0	0	0	0	0	
Cyproheptadine	x	x	0	0	0			
Amitriptyline	x	x	0	0	0	0		
Methyl-DOPA	x	x	x	0	0	x[d]		
Penicillamine	x	x	x	x	0			
Ethacrynic acid	x	x	x	x	0	0	0	
Protriptyline HCl	x	0	0	0	0	0	0	
Thiabendazole	x	x	x	x	0	0	0	0
Dexamethasone	x	x	x	0	0			

[a] By physical examination or hematologic, biochemical, and/or anatomical studies.

[b] x — Toxicity demonstrated.

[c] 0 — Continuing duration of study, no additional toxicity.

[d] Additional finding of precipitate in kidneys. No earlier sacrifice, so time of onset not known. Found in rats but not in dogs or monkeys.

Source: Adapted from Peck, 1968 — An Appraisal of Drug Safety Evaluation in Animals and the Extrapolation of Results to Man. In: Importance of Fundamental Principles in Drug Evaluation, D. H. Tedeschi and R. E. Tedeschi, eds. Raven Press, New York. pp. 450-471. Reprinted with permission of the publisher.

the idea that shorter tests can provide reasonable indications of long-term toxicity.

In contrast to the papers by Weil, Hayes (1972) stated that although the 90-day or 70-exposure test is sufficient to determine most long-term effects, reduction to lesser durations is not adequate. He recommended this after consideration of some data on chemosterilants.

Initially Hayes (1967*b*) felt that some drugs could be tested in 30 exposures, but on reexamination he preferred the 90-day exposure test. Thus, there is definitely some opposition to reduction of test duration.

McNamara (1976) compiled data similar to Weil and McCollister (1963) from the literature using 82 studies evaluating 122 compounds with a variety of test species. Despite the variability due to differences in species and experimental design, McNamara's data agree with that of Weil and McCollister in recommending that 2-year studies need not always be performed. The results are summarized in Table 4.14 in which formulas are given designating the safe long-term dose levels predicted by short-term tests. As with the other authors, McNamara qualified this predictive value to exclude carcinogenetic and teratogenetic effects. In conclusion he stated:

Table 4.14. Prediction of long-term no-effect doses

Dose	Time in which repeated dosage will produce no effect
$LD_{50}/100$	3 months
$LD_{50}/1000$	Lifetime
3-month no-effect dose/10	Lifetime

Source: Adapted from McNamara, 1976. Reprinted with permission of the publisher.

> If any toxic effect occurs in 3 months, additional (and perhaps more serious) effects may appear with continued dosing. If no effect occurs in 3 months, there is a low likelihood that any effect will occur on continued dosing for 1 year. Thus, these dose relationships can be of great value in decreasing the time involved in LT toxicological testing. Experimental evidence strongly supports the view that LT no-effect doses can be reliably predicted from ST studies.

4.3.3 Comparison Summaries

Gaunt et al. (1965) studied selected aspects of the oral toxicity of butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) in the rat with dose levels of 0.1% for 16 weeks. Based on previous studies, the authors concentrated on liver effects, although other toxicity parameters were monitored. As shown in Table 4.15, the principal effects were increases in ascorbic acid excretion, liver weight, and adrenal weight. For BHT, the females showed significant changes in ascorbic acid excretion and liver weight by the 4th week, but the adrenal weight effects did not appear at a significant level until the 12th week. In the male rat, a trend of increasing ascorbic acid excretion for BHT was noted from the 4th week on, but not always at a significant level. The same was true for increases in liver and adrenal weights and in general for all parameters of the BHA studies in both sexes. In this study, the 12-week duration definitely predicted the longer-term effects of 16 weeks. The 4-week duration did provide good indications of the toxicity trends but not always at a significant level.

In a 90-day study of dibutyl(diethylene glycol bisphthalate) (DDGB), a plasticizer, Hall, Austin, and Fairweather (1966) tested the oral toxicity in rats. As shown in Table 4.16, the effect on body weight gain was evident by the fourth week when a significant decrease was noted for males at the high (2.5% of diet) and intermediate (1.0% of diet) doses, and for females at the high dose. The only other toxic effect noted was an increase in relative liver, heart, and brain weights after 90 days. The authors felt that these changes were due to the body weight loss since the absolute organ weights did not change. Therefore, in this case, the only toxic effect attributable to DDGB would have been detected by a 4- or 8-week test.

Misu et al. (1966) tested Sumithion, an insecticide, for 90 days given in the diet of the rat at concentrations of 32, 63, 125, 250, and 500 ppm. Toxic effects began to appear at the high dose during the first week of testing: the mean body weight was decreased (Fig. 4.1), the testes and brain weights increased (Table 4.17), and the cholinestrase activity decreased (Fig. 4.2). The growth rate decrease was most noticeable after the first week, but began to parallel the control values as

Table 4.15. Urinary ascorbic acid excretion and relative liver and adrenal weights of rats fed butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) at 0.1% of the diet for 16 weeks[a]

Compound	Duration of feeding (week)	Ascorbic acid[b] (mg/kg/day)		Organ weight to body weight ratio (%)			
				Liver		Adrenal	
		Male	Female	Male	Female	Male	Female
Arachis oil, control	4	2.20 ± 0.40	3.25 ± 0.35	4.41 ± 0.31	3.94 ± 0.08	0.010 ± 0.0008	0.016 ± 0.0005
BHT		4.74 ± 0.98	7.81 ± 1.27	4.68 ± 0.18	4.35 ± 0.08	0.013 ± 0.0010	0.018 ± 0.0008
BHA		1.84 ± 0.24	4.36 ± 0.48	4.36 ± 0.17	4.70 ± 0.12	0.012 ± 0.0007	0.018 ± 0.0011
Arachis oil, control	8	4.19 ± 1.08	3.28 ± 0.08	4.13 ± 0.03	3.71 ± 0.11	0.010 ± 0.0005	0.018 ± 0.0011
BHT		6.90 ± 1.26	3.49 ± 0.31	4.18 ± 0.06	4.00 ± 0.09	0.010 ± 0.0004	0.015 ± 0.0009
BHA		4.89 ± 1.56	3.67 ± 0.31	5.25 ± 0.17	3.82 ± 0.12	0.010 ± 0.0011	0.017 ± 0.0006
Arachis oil, control	12	2.10 ± 0.21	4.12 ± 0.57	4.53 ± 0.08	3.13 ± 0.10	0.009 ± 0.0017	0.017 ± 0.0008
BHT		3.41 ± 0.41	4.81 ± 0.61	4.93 ± 0.07	3.73 ± 0.08	0.010 ± 0.0026	0.022 ± 0.0006
BHA		1.87 ± 0.17	2.32 ± 0.36	4.58 ± 0.14	3.60 ± 0.06	0.011 ± 0.0008	0.020 ± 0.0009
Arachis oil, control	16	2.30 ± 0.34	0.98 ± 0.14	4.30 ± 0.10	3.21 ± 0.10	0.009 ± 0.0081	0.016 ± 0.0014
BHT		3.13 ± 0.41	2.17 ± 0.17	4.73 ± 0.08	3.61 ± 0.10	0.010 ± 0.0061	0.019 ± 0.0009
BHA		2.29 ± 0.24	0.92 ± 0.17	4.72 ± 0.09	3.50 ± 0.17	0.011 ± 0.0005	0.020 ± 0.0004

[a] Each result represents the mean ± statistical error for six animals.

[b] Ascorbic acid is the mean of four days' excretion.

[c] $P < 0.05$.

[d] $P < 0.01$.

[e] $P < 0.001$.

Source: Adapted from Gaunt et al., 1965. Reprinted with permission of the publisher.

Table 4.16. Mean values of body weights, food consumption, and dibutyl(diethylene glycol bisphthalate) (DDGB) intake of rats fed DDGB at 0% to 2.5% of the diet for 11 weeks[a]

Dietary level (%)	Body weight in grams at end of week				Food consumption in grams per rat per day at end of week				DDGB intake[b] in grams per kg per day at end of week			
	0[c]	4	8	11	0[c]	4	8	11	0[c]	4	8	11
					Male							
0.0	96	241	317	352	11.9	23.4	18.7	18.0				
0.25	100	226	301	335	11.6	20.5	20.2	19.4	0.29	0.23	0.17	0.14
1.0	91	214[d]	280[e]	315[e]	9.5	19.3	17.1	16.7	1.04	0.90	0.61	0.53
2.5	98	170[e]	232[e]	262[e]	5.8	16.3	15.5	14.9	1.48	2.40	1.68	1.41
					Female							
0.0	82	169	205	220	9.5	15.7	13.9	14.5				
0.25	82	156	185[d]	199[e]	9.7	14.5	13.1	11.3	0.30	0.23	0.18	0.14
1.0	87	160	196	211[d]	9.5	14.6	13.5	13.5	1.09	0.91	0.69	0.64
2.5	83	145[e]	177[e]	190[e]	5.6	14.2	13.6	11.4	1.67	2.44	1.93	1.52

[a] Values are the means for groups of 10 animals.

[b] DDGB intakes are calculated from data on body weight and food consumption.

[c] Day one of feeding.

[d] $P < 0.05$.

[e] $P < 0.001$.

Source: Adapted from Hall, Austin, and Fairweather, 1966. Reprinted with permission of the publisher.

the exposure period progressed. However, the final body weight was still significantly below control values. Testes weight was significantly lower by the 11th day and remained so until the 90th day. The same pattern was found for the cholinestrase activity in the liver, kidney, brain, and red blood cells. In each case the initial stress was the greatest, producing the most effect. All toxic effects showed recovery or stabilization at the 90th day. Some minor effects of the same type as occurred at the high dose were noted with a dose of 250 ppm, and the no-effect level was 125 ppm. Thus, it appears that all of the toxic effects could have been detected by an 11- or 30-exposure test, and only the stabilization trend would have been missed.

In a study of the oral toxicity of diuron, a herbicide, Hodge, Downs, Panner, Smith, Maynard, Clayton, and Rhodes (1967) utilized rat tests with durations of 1 month, 3 months, or 2 years. The dose levels employed were: 200, 400, 2000, 4000, and 8000 ppm (1 month); 50, 250, 500, 2500, and 5000 ppm (3 months); and 25, 125, 250, and 2500 ppm

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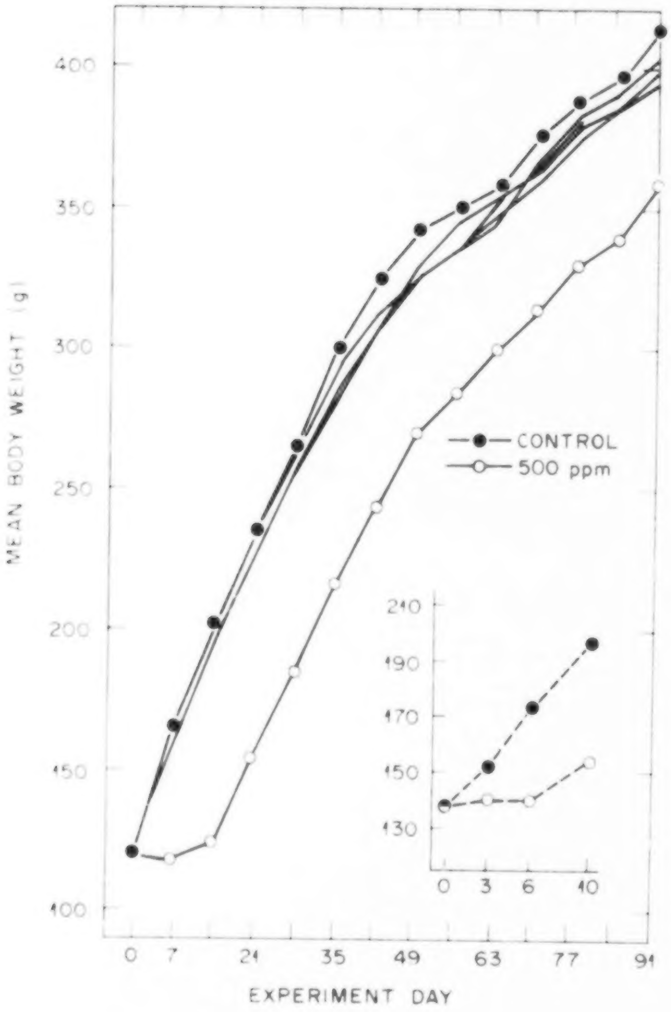

Fig. 4.1. Changes in the mean body weight of rats fed Sumithion in the diet for 90 days. Source: Adapted from Misu et al., 1966. Reprinted with permission of the publisher.

(2 years). Depression of the growth rate occurred at 1 month (2000, 4000, and 8000 ppm), at 3 months (2500 and 5000 ppm), and at 2 years (2500 ppm). Mortality was evident at 1 month (6 of 10 rats at 8000 ppm) and at 2 years (44 of 70 rats at 2500 ppm), but no mortality was seen at 3 months. However, the mortality was affected by disease vectors, and the authors concluded that the death trend in the 2-year study was not due to diuron levels. The red blood cell counts were decreased at

Table 4.17. Mean testes and brain weights, as percentage of body weight, of control rats and rats fed a dietary level of 500 ppm of Sumithion for various periods of time

Organ	Organ weight after days of feeding indicated							
	11		30		60		90	
	Control (%)	500 ppm (%)	Control (%)	500 ppm (%)	Control (%)	500 ppm (%)	Control (%)	500 ppm (%)
Left testis	0.41 ± 0.02	0.53[a] ± 0.02	0.40 ± 0.02	0.60 ± 0.40	0.29 ± 0.02	0.38 ± 0.01	0.27 ± 0.02	0.35 ± 0.02
Right testis	0.42 ± 0.02	0.52[a] ± 0.01	0.40 ± 0.02	0.59 ± 0.01	0.29 ± 0.02	0.39 ± 0.01	0.26 ± 0.02	0.34 ± 0.03
Brain stem	0.21 ± 0.00	0.25[b] ± 0.01	0.15 ± 0.04	0.23 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.15 ± 0.01
Brain cortex	0.58 ± 0.02	0.79[a] ± 0.01	0.46 ± 0.01	0.54 ± 0.12	0.32 ± 0.01	0.40 ± 0.02	0.30 ± 0.01	0.33 ± 0.01

[a] Value differs from controls, $P < 0.01$.

[b] Value differs from controls, $0.01 < P < 0.05$.

[c] Value differs from controls, $0.05 < P < 0.5$.

[d] Not significant, $P > 0.5$.

Source: Adapted from Misu et al., 1966. Reprinted with permission of the publisher.

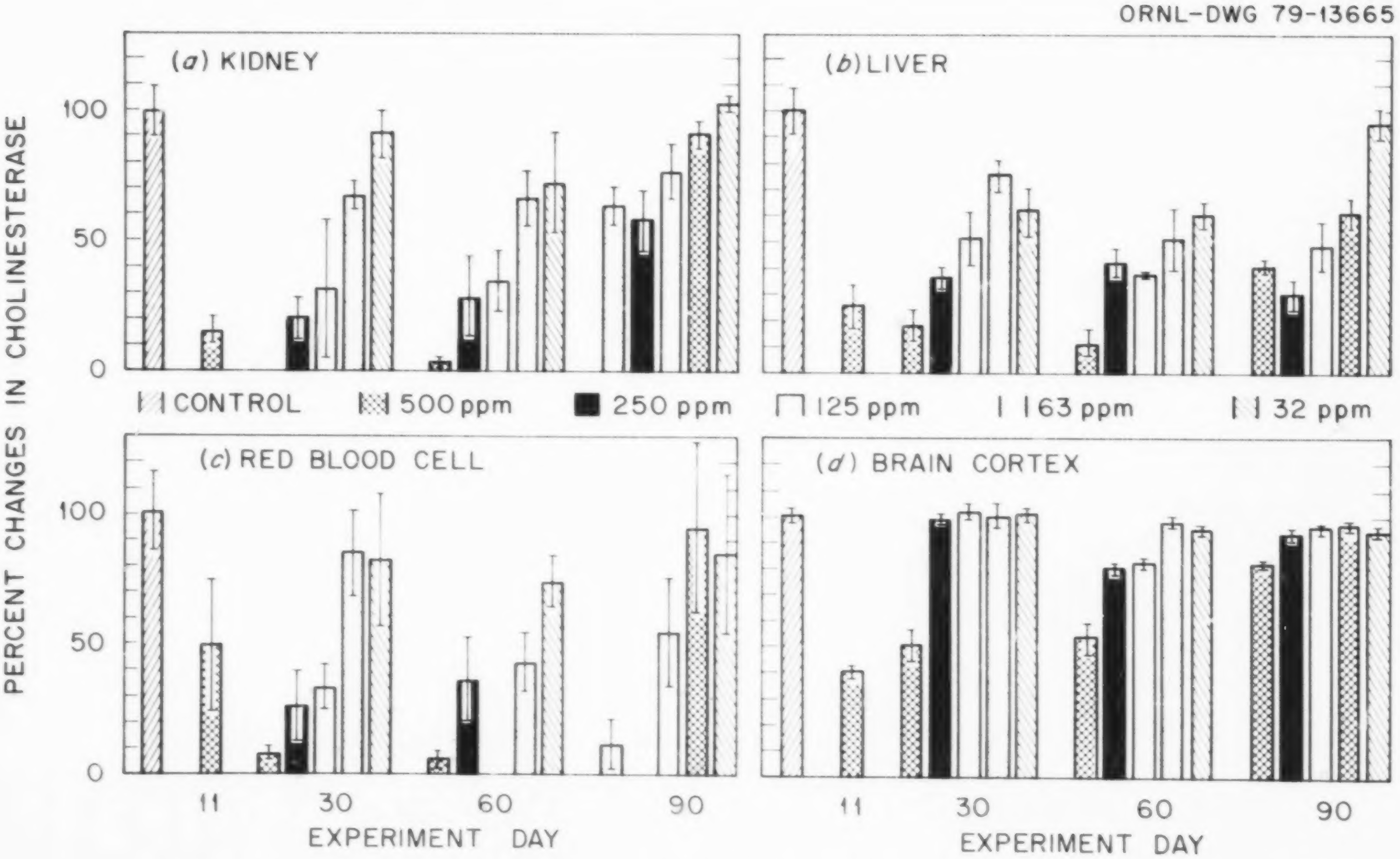

Fig. 4.2. Changes in cholinesterase activity in the kidney, liver, red blood cells, and brain cortex of rats fed Sumithion for 90 days. Source: Adapted from Misu et al., 1966. Reprinted with permission of the publisher.

1 month (4000 and 8000 ppm), at 3 months (2500 and 5000 ppm), and at 2 years (2500 ppm). The hemoglobin concentrations were also depressed at 1 month (4000 and 8000 ppm), at 3 months (5000 ppm), and at 2 years (2500 ppm). The 1-month study indicated a decrease in hematocrit that was not evident in the longer studies. Organ weights were also affected. After 1 month at 8000 ppm, the liver and kidney weights were decreased, and the spleen weight was increased. At 3 months the spleen weight was increased (5000 ppm), but the liver and kidney weights were unaffected. In the 2-year study the spleen weight was increased (2500 ppm) at 9 and 17 months, but not at 24 months. Histopathologic data were not significantly different from the controls for all tests regardless of duration. In conclusion, it appears that the major toxic effects were sufficiently indicated by the 1-month duration. Extension to 3 months and 2 years provided little additional information.

Ambrose et al. (1972) studied 3'-4'-dichloropropionanilide in rats after 13 weeks and 2 years of oral exposure. In the 13-week study, dose levels of 100, 330, 1000, 3300, 10 000, and 50 000 ppm were used, and the 2-year study used levels of 100, 400, and 1600 ppm. The significant effects recorded after 13 weeks of dosing included growth depression (high and intermediate doses), a decrease in hemoglobin levels (high and intermediate doses), and increases in weight for the heart (high dose), spleen and liver (females at high and intermediate doses), and testes (high dose). After the 2-year exposure period, the effects at the high dose included growth rate depression, increased female liver and kidney weights, and increased testes weight. These were all detected with the 13-week study. The hemoglobin level was also depressed at the high dose in the 2-year study, but, in contrast to the 13-week study, results occurred only in the female rats. Thus, in this study there was a good prediction of chronic (2-year) effects from the 13-week data, suggesting that the 13-week study would have been sufficient.

Lawrence et al. (1972) studied epichlorohydrin in the rat by intraperitoneal injection for durations of 30 daily injections (with doses of 0.00955 and 0.01910 mL/kg) or 12 weeks of three injections per week (with doses of 0.00955, 0.01910, and 0.04774 mL/kg). In the cumulative toxicity test (30 daily exposures), weight gain was significantly decreased at the high dose by day 15 and at the low dose by day 20 (Table 4.18). In the 12-week study, the high dose exhibited a significant decrease in weight gain until the last week (Table 4.19). The hematology and biochemical parameters showed no significant trends in most tests. However, hemoglobin levels did show a significant decrease at the high dose at the end of the cumulative toxicity test and a dose-dependent decrease after the 12-week study. The measurement of organ weights also showed some changes attributed to toxic effects. In the 30-day study, the kidney weight decreased significantly at both doses (Table 4.20). After 12 weeks of dosing at the high dose, the kidney, liver, and heart all showed significant weight increases, and the brain showed a significant weight decrease (Table 4.21). In comparison of these two experiments, the total number of injection exposures is similar (30 vs 36), but the duration of exposure is not (4 weeks vs 12 weeks). The results from both studies are quite similar, which suggests either that the shorter duration adequately predicts the results of the longer duration or that the number of doses given is the key factor in a repeated-dose study.

Table 4.18. Cumulative toxicity of epichlorohydrin: body weight gain in grams (mean ± statistical error)

Day	Cottonseed oil control	Epichlorohydrin	
		0.00955 mL/kg	0.01910 mL/kg
5	28.17 ± 1.96	36.25 ± 5.73	26.00 ± 1.54
10	66.00 ± 2.44	71.92 ± 6.15	63.67 ± 2.68
15	111.25 ± 3.39	105.25 ± 7.24	91.33 ± 4.33[a]
20	145.67 ± 3.75	123.42 ± 4.83[a]	131.58 ± 2.87[a]
25	157.25 ± 4.38	145.67 ± 6.44	153.92 ± 7.33
30	183.33 ± 5.59	167.67 ± 7.27	158.50 ± 6.82[b]

[a] Significantly different from controls at 99% level ($P = 0.01$) by Student's t test.

[b] Significantly different from controls at 95% level ($P = 0.05$) by Student's t test.

Source: Adapted from Lawrence et al., 1972. Reproduced with permission of the copyright owner.

Table 4.19. Subacute toxicity of epichlorohydrin: body weight gain in grams (mean ± statistical error)

Week	Cottonseed oil control	Epichlorohydrin		
		0.0095 mL/kg	0.0190 mL/kg	0.04774 mL/kg
1	46.58 ± 2.88	38.58 ± 4.50	41.42 ± 2.40	24.00 ± 3.14[a]
2	76.50 ± 6.15	94.42 ± 3.05[b]	86.50 ± 5.14	55.09 ± 10.23
3	105.33 ± 5.86	119.83 ± 3.72[b]	111.83 ± 6.79	97.00 ± 9.27
4	186.17 ± 5.74	193.42 ± 4.74	175.58 ± 11.02	128.30 ± 11.54[a]
5	214.25 ± 8.14	228.08 ± 6.80	203.50 ± 10.73	172.00 ± 11.37[b]
6	254.83 ± 6.52	254.33 ± 11.07	242.75 ± 11.44	195.60 ± 10.29[a]
7	279.25 ± 7.16	267.08 ± 9.11	261.25 ± 12.27	218.50 ± 11.14[a]
8	296.17 ± 7.10	289.92 ± 5.98	276.00 ± 11.47	246.80 ± 12.16[a]
9	333.50 ± 9.72	330.75 ± 9.59	315.67 ± 21.92	266.60 ± 13.19[a]
10	345.50 ± 13.49	359.83 ± 9.64	334.00 ± 13.81	293.70 ± 14.19[b]
11	356.67 ± 14.75	370.42 ± 10.82	371.17 ± 12.08	286.70 ± 21.91[b]
12	358.92 ± 13.27	383.42 ± 11.22	380.92 ± 13.34	328.33 ± 18.19

[a] Significantly different from controls at 99% level ($P = 0.01$) by Student's t test.

[b] Significantly different from controls at 95% level ($P = 0.05$) by Student's t test.

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In two companion studies, Kociba et al. (1976; 1978) evaluated the toxicity potential of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) administered in the diet of rats for 13 weeks (1.0 and 0.1 μg/kg) and 2 years (0.1 and 0.01 μg/kg). As shown in Table 4.22 the results were quite similar for mortality, body weight gain, alkaline phosphatase, packed cell volume, red blood cell count, hemoglobin concentration, relative weights of the liver and thymus, and histopathologic changes in the liver, lung, thymus, uteri, and lymphoid tissues. Differences between the two studies consisted mainly of the more transient effects indicated by the subchronic study, with only a few different effects indicated by the chronic study. Although there were minor differences in serum enzyme levels, hematology values, and relative organ weight changes, the subchronic study did evaluate the major effects found in the chronic study.

Table 4.20. Cumulative toxicity of epichlorohydrin: percent organ weight to body weight of rats (mean ± statistical error)

Organ	Cottonseed oil control	Epichlorohydrin	
		0.00955 mL/kg	0.01910 mL/kg
Adrenals	0.014 ± 0.001	0.014 ± 0.001	0.013 ± 0.002
Brain	0.475 ± 0.017	0.567 ± 0.041	0.543 ± 0.033
Gonads	0.975 ± 0.037	0.995 ± 0.051	0.971 ± 0.037
Heart	0.273 ± 0.006	0.309 ± 0.022	0.378 ± 0.078
Kidneys	0.654 ± 0.023	0.750 ± 0.025[a]	0.786 ± 0.035[a]
Liver	3.960 ± 0.140	4.150 ± 0.230	3.916 ± 0.056
Lungs	0.381 ± 0.027	0.409 ± 0.028	0.407 ± 0.015
Spleen	0.236 ± 0.010	0.266 ± 0.017	0.253 ± 0.012

[a] Significantly different from controls at 95% level ($P = 0.05$) by Student's t test.

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Table 4.21. Subacute toxicity of epichlorohydrin: percent organ weight to body weight of rats[a] (mean ± statistical error)

Organ	Cottonseed oil control	Epichlorohydrin		
		0.0095 mL/kg	0.0190 mL/kg	0.04774 mL/kg
Adrenals	0.023 ± 0.013	0.011 ± 0.001	0.010 ± 0.001	0.013 ± 0.002
Brain	0.444 ± 0.018	0.426 ± 0.012	0.439 ± 0.029	0.324 ± 0.027[b]
Gonads	0.785 ± 0.028	0.707 ± 0.034	0.866 ± 0.054	[c]
Heart	0.291 ± 0.011	0.302 ± 0.018	0.306 ± 0.030	0.402 ± 0.029[b]
Kidneys	0.670 ± 0.034	0.636 ± 0.032	0.728 ± 0.049	0.917 ± 0.054[b]
Liver	2.983 ± 0.219	3.232 ± 0.157	3.276 ± 0.186	4.070 ± 0.276[d]
Spleen	0.174 ± 0.012	0.194 ± 0.010	0.208 ± 0.031	0.225 ± 0.021

[a] Calculated as (organ weight, grams per gram of body weight) × 100 = percent organ weight to body weight.

[b] Significantly different from controls at 99% level ($P = 0.01$) by Student's t test.

[c] Gonadal weights were not determined in this group.

[d] Significantly different from controls at 95% level ($P = 0.05$) by Student's t test.

Source: Adapted from Lawrence et al., 1972. Reproduced with permission of the copyright owner.

Table 4.22. Comparison of 13-week and 2-year
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) studies

Test parameters with significant changes	13-week study[a]		2-year study[b]	
	1.0 μg/kg	0.1 μg/kg	0.1 μg/kg	0.01 μg/kg
Mortality	F[c]		F	
Body weight	↓[d]	↓	↓	↓F
Food consumption	↓	↓		
Packed cell volume	↓M; ↑F	↓M	↓F	
Red blood cell count	↓M; ↑F	↓M	↓M	
Hemoglobin concentration	↓M; ↑F	↓M	↓	
Reticulocyte count	↑			
Thrombocyte count	↓			
Total leucocyte count	↑F			
Total and direct bilirubin	↑	↑F		
Glutamic-pyruvic transaminase			↑F	
Alkaline phosphatase	↑	↑F	↑F	
Relative thymus weight	↓	↓	↓F	
Relative liver weight	↑	↑	↑F	↑F
Histopathologic changes[e] in:				
Thymus	M, F	M, F	F	
Other lymphoid tissue	M, F	M, F		
Uteri	F		F	
Ovaries	F			
Liver	M, F	M, F	M, F	M, F
Lung	M, F		M, F	F
Brain			F	
Pituitary			F	

[a] Adapted from Kociba et al., 1976.

[b] Adapted from Kociba et al., 1978.

[c] F — females only; M — males only.

[d] ↓ — decrease in value vs control; ↑ — increase in value vs control.

[e] Histopathologic changes exclusive of carcinogenic tumors.

In another study of TCDD, Harris et al. (1973) tested the toxic effects in female rats from gastric intubation for 31 consecutive days with doses of 0.1, 1, and 10 mg/kg. At the high dose, significant weight loss (Table 4.23) occurred by the 7th day, and 15 of 16 rats died by a mean of 21.8 days after the start of dosing. For the intermediate dose, weight gain was depressed significantly during the first 35 days, but reversed to yield significant growth increases during a recovery period on days 35 to 63. Organ weight changes occurred only in the liver and the thymus (Table 4.24). Liver weight increased at all three dose levels (although statistically erratic) by the 10th day of exposure. Livers at the high dose showed a decrease in weight at the 17th and 24th

Table 4.23. Weight gain of female rats receiving 31 daily doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)

Daily TCDD dose (μg/kg)	Initial number of animals	Initial body weight (g ± standard deviation)	Weight gain (g ± standard deviation)		
			Days 1 to 7	Days 1 to 35[a]	Days 35 to 63[a]
0	16	183.3 ± 6.28	15.8 ± 6.6	65.8 ± 8.0	28.4 ± 9.7
1	12	185.6 ± 5.04	12.1 ± 6.4	34.8 ± 12.2[b]	42.0 ± 11.9[c]
10	16	184.0 ± 8.00	-21.4 ± 14.9[b]		

[a] Values based on 5 and 8 rats at 0 and 1 μg/kg respectively; 15 of 16 rats died or were killed when moribund at mean of 21.8 days.

[b] $P < 0.01$.

[c] $P < 0.06$.

Source: Adapted from Harris et al., 1973.

Table 4.24. Liver and thymus weights of female rats receiving daily doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)

Number of doses	Number of animals	0 μg/kg		0.1 μg/kg		1.0 μg/kg		10 μg/kg	
		Liver (g ± SE[a])	Thymus (mg ± SE)[b]	Liver (g ± SE)	Thymus (mg ± SE)[b]	Liver (g ± SE)	Thymus (mg ± SE)[b]	Liver (g ± SE)	Thymus (mg ± SE)[b]
3	4	7.80 ± 0.48		8.62 ± 0.41		9.32 ± 0.45		9.58 ± 0.43	
6	4	8.41 ± 0.36		10.35 ± 0.45[c]		9.00 ± 0.14		10.84 ± 0.97	
10	4	9.14 ± 0.21		10.78 ± 0.41[c]		11.53 ± 0.40[d]		10.53 ± 0.36[c]	
13	4	7.90 ± 0.31	530 ± 48	10.17 ± 0.94[c]	492 ± 81	9.76 ± 0.62	390 ± 58	9.80 ± 1.08	142 ± 12[c]
17	4	9.26 ± 0.42	615 ± 59	10.91 ± 0.37[c]	468 ± 22	11.33 ± 0.29[d]	270 ± 11[d]	7.52 ± 1.37	110 ± 48[d]
24	4	9.45 ± 0.31	525 ± 43	11.40 ± 1.05	412 ± 28[c]	11.39 ± 0.98	372 ± 28[c]	4.67 ± 0.21[d]	32 ± 10[d]
31	4	9.70 ± 0.49	558 ± 87	11.78 ± 0.81	475 ± 49	12.84 ± 0.84[c]	260 ± 41[d]		

[a] Statistical error.

[b] All dose response tests for thymus significant at $P < 0.01$.

[c] $P < 0.05$.

[d] $P < 0.01$.

Source: Adapted from Harris et al., 1973.

days of exposure, caused primarily by reduced body weight. The thymus weight was significantly decreased by day 17 at both the high and intermediate doses. Results of this study indicated that the 30-day duration would have been sufficient to detect body and organ weight changes and that even 24 days would have indicated the majority of effects. The results of this 30-day study also correlate relatively well with the results of the longer 13-week and 2-year TCDD toxicity studies by Kociba et al. (1976; 1978).

Rosenkrantz et al. (1975) assessed the oral toxicity of Δ^9-tetrahydrocannabinol, the major active ingredient in marijuana, in rats exposed for 28, 90, and 180 days at concentrations of 2, 10, and 50 mg/kg per day. Body growth depression was one of the primary effects, with an 8% to 12% rate decline at 28 and 90 days, increasing to 9% to 17% by 180 days. Hematological and biochemical parameters showed several trends. At 28 days, although there were no statistically significant changes, there were increases in lymphocyte levels of males, increases in reticulocyte levels of females, and decreases in polymorphonuclear cells of males. At 90 days, red blood cell numbers increased 11%, hematocrit levels increased 8%, and for females the white blood cell levels increased 19% to 74% in a dose-dependent manner. At 180 days, hematocrit and red blood cells rose 11% and 7%, respectively, in the females. Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) levels rose in the males at rates of 44% and 65%. The relative adrenal weight increased 30% at 28 days, and for males the pancreas weight increased 10% and the prostate weight decreased 30%. After 90 days of treatment, the brain weight increased 3% to 25%, and the kidney weight increased 7% to 18%. Adrenal weight remained elevated while the prostate weight continued to decrease, but only at 6% to 21%. At 180 days, more organs revealed weight changes including increases in the brain (16%), lung (10% to 23%), kidney (11% to 20%), heart (12%), liver (10%), and adrenal glands (34% to 56%). Changes in organ weight in the males were as follows: the thymus decreased 17%, the pancreas increased 96%, the prostate increased 13%, and the testes increased 25%. In the females the weight of the uterus decreased 14%. Although many of these changes at 180 days were dose dependent, the significance was borderline. None of the histopathological changes was considered significant, with lesions occurring only incidentally in both control and treatment animals. In this study, the 28-day duration revealed the same changes as the longer studies for growth rate depression and weight changes in minor organs. However, the study failed to detect weight changes in major organs and in hematology values and suggests that a 28-day test does not adequately predict what happens at 90 days. By the 90th day, toxic effects in these categories were detected. Although the 180-day duration revealed more changes, the indication of toxicity was sufficient at 90 days to suggest the potential danger of the compound.

In a study of the oral toxicity of fominoben-HCl, Kast et al. (1975b) dosed rats daily for 1 and 6 months. Even though the 6-month test is not a subchronic duration, this study is useful for evaluating the effectiveness of short-term tests (less than 90 days) to predict long-term effects. In the 1-month study, dose levels of 250, 500, 1000, and 2000 mg/kg were used, and the 6-month study used levels of 50, 100, and 200 mg/kg. In the shorter test, mortality was evident by the second week at the high dose, with 9 of 30 rats dying. Body weight gain was depressed from the first week until the end of the 1-month test. Other effects detected at the high dose were increases in SGOT, SGPT, serum alkaline phosphatase, serum bilirubin, and serum cholesterol; a decrease in total protein; liver pathology; atrophy of the gastric mucosa; and decreases in thymus and testes weights in male rats. The liver weight increased at a dose-dependent rate. In the 6-month experiment, no mortality occurred, but body weight gain was decreased at the high dose. The biochemical and hematological parameters were affected less in the longer study than in the 1-month study, with increases in SGOT and SGPT activities being the only significant changes. The pathology was limited to liver damage at the high dose, and liver weight increased at a dose-dependent rate. In comparing the results of the two duration periods, it is apparent that the shorter test indicated the major effects, particularly the liver damage, that were revealed in the 6-month study. Therefore, in this study, the shorter test would have been a sufficient predictor of long-term effects.

In another study by Kast et al. (1975*a*) the same experimental design was used to test the toxicity of fenoterol-HBr. The doses used were 15, 150, and 1500 mg/kg (1 month) and 3, 30, and 300 mg/kg (6 months). In the shorter study, mortality at the high dose was high (64% for males, 24% for females), although in the chronic study only one substance-related death occurred. The body weight gain was significantly decreased at the high dose in the 1-month test, but showed a significant increase in the female after 6 months of testing. The hematological and biochemical tests performed for the 1-month duration revealed, at the high dose, decreases in platelet count (also at intermediate dose) and blood glucose levels and increases in blood urea nitrogen, Ca$^+$, SGOT, and SGPT levels. In the 6-month study, an alkaline phosphatase increase at the high dose was the only significant change. although blood glucose levels showed a slight depression. Organ weights increased for the salivary glands (high dose), heart (high and intermediate doses), and liver (females at high and intermediate doses). However, the males showed a decrease in liver and testes weights at the high dose. In the 6-month study, only female heart weight increased at high and intermediate doses. In the shorter test, histopathological examination revealed salivary gland and heart (primarily ischemic lesions) damage at the high dose. Testes were also atrophied in three males at the high dose. In the 6-month test, only the heart damage (again with a predominance of ischemic lesions) occurred at the high dose. Comparing the two experiments, it appears the shorter test indicated more toxicity than the longer evaluation. This may be caused by different dose magnitudes or the effects of fenoterol-HBr being acute in nature, or it may indicate an adaptation to the toxic effects which reduced the final effects in the chronic study.

Kruysse et al. (1977) studied the inhalation toxicity of peroxyacetyl nitrate (PAN) by exposing the rat for 4 and 13 weeks. The exposure period was 6 h per day, 5 days per week, with dose levels of 0.9, 4.1, and 11.8 ppm (4 weeks) and 0.2, 1.0, and 4.6 ppm (13 weeks). In a comparison of the effects of the two durations, both produced quite similar results, especially at the high doses. In the 4-week test, mortality was observed at the high dose, with 9 of 20 rats dying. No mortality was seen after the 13-week exposure. Growth depression occurred at the high dose (4 and 13 weeks) and irregularly at the intermediate dose (4 weeks). This was accompanied in both tests by reduced food consumption. In the 4-week test (high dose) hemoglobin, hematocrit, and red blood cell counts were all significantly elevated; in the 13-week test (high dose) hemoglobin and neutrophil levels were increased and lymphocyte levels decreased. Relative organ weights were also affected, but the authors stated that this may be due to severe growth depression, because absolute organ values were below control values. After 4 weeks at the high dose, heart, kidney, and lung weights increased, and the spleen weight decreased. After 13 weeks at the high dose, testes, thyroid, and lung weights all increased. Histopathology revealed lung and tracheal damage after 4 and 13 weeks at the high and intermediate doses. The degree and depth of damage increased with higher doses. The authors concluded that "comparable PAN effects" were observed at the high dose in 4- and 13-week tests. It appears that the 4-week test would be sufficient in this case.

Gray et al. (1977) studied the toxicity of di-(2-ethylhexyl)phthalate, a plasticizer, in the diet of the rat for 17 weeks at concentrations of 0.2%, 1.0%, and 2.0%. Toxic effects were evident early in the study, particularly at the high dose. Loss of body fur was noticeable by the 1st week at the high dose and continued until the 17th week, with the affected rats quite emaciated at that time. Decreases in the body growth rate (Table 4.25) appeared by the 2nd day at the high dose and at the intermediate dose by day 6 in the males. This decrease was caused in part by lowered food consumption, which was especially prominent during the first 48 h. As shown in Table 4.26, there were irregular fluctuations in the packed cell volume, hemoglobin, and red blood cell values throughout the study. The decrease in packed cell volume was the only significant change occurring at both 6 and 17 weeks. As shown in Table 4.27, changes in relative organ weights occurred by the 2nd week of dosing. Increased weights were noted for the brain (females), liver, stomach, small intestine (males), caecum (males), thyroid (males), and adrenals. Decreases in weight were noted for the heart, spleen, and testes. At 6 weeks, the only additional changes were an increase in

Table 4.25. Mean body weights and water intake of rats fed diets containing 0% to 2.0% di-(2-ethylhexyl)phthalate (DEHP) for up to 17 weeks

Dietary level (%)	Body weight[a] in grams at day						Water intake[b] in ml per rat per day at day						Mean water intake (ml per rat per day)
	0[c]	1	27	55	90	120	0[c]	1	27	55	90	120	
							Male						
0.0	96	103	340	478	569	628	18.3	18.5	37.1	38.0	28.5	26.3	30.1
0.2	98	105	325	455	539	588	17.6	19.7	37.3	36.3	32.1	24.7	30.1
1.0	98	100	297[d]	417[d]	493[d]	546[e]	18.0	15.1	34.3	27.9[d]	29.0	27.7	27.8[f]
2.0	99	99	187[e]	300[e]	413[e]	447[e]	18.4	13.7	24.9[e]	30.9	34.4	26.9	25.7[e]
							Female						
0.0	85	92	214	273	309	329	15.9	15.7	21.5	21.9	18.1	19.4	22.3[f]
0.2	88	95	216	277	308[f]	325	17.9	18.9	24.9[f]	34.5	26.5[f]	22.1	25.0[f]
1.0	87	90	210	259	286[d]	297[e]	17.4	15.4	26.6[d]	24.5	23.9[d]	22.6	24.3[f]
2.0	88	87	131[e]	164[e]	191[e]	201[e]	18.1	14.8	21.1	21.3	18.9	16.7	19.7

[a] Body weights are the means for 15 animals.

[b] Values for water consumption are the means for three cages of five animals and were measured over the 24-h period preceding the day shown.

[c] First day of treatment.

[d] $P < 0.01$.

[e] $P < 0.001$.

[f] $P < 0.05$.

Source: Adapted from Gray et al., 1977. Reprinted with permission of the publisher.

Table 4.26. Mean hematological values[a] for rats fed diets containing 0% to 2.0% di-(2-ethylhexyl)phthalate (DEHP) for 2, 6, or 17 weeks

Sex and dietary level (%)	Number of rats	Hb[b] (g per 100 ml)	PCV[c] (%)	RBC[d] (10^6 per mm^3)	Retics[e] (% of RBC)	WBC[f] (10^3 per mm^3)
			Week 2			
Male						
0	5	14.8	45	6.26	2.0	7.2
1.0	5	13.0[g]	40	5.77[g]	2.7	5.6
2.0	5	14.8	42	6.48	1.3	5.3
Female						
0	5	15.1	43	6.50	1.6	4.9
1.0	5	14.4	38	6.74	2.2	4.2
2.0	5	14.5	41	6.82	0.8[h]	6.4
			Week 6			
Male						
0	5	15.1	48	7.08	1.4	6.2
1.0	5	15.0	41	6.96	1.6	6.0
2.0	5	14.9	46	6.86	1.3	5.0
Female						
0	5	15.8	49	7.81	1.2	6.9
1.0	5	15.4	44	7.90	1.0	4.7
2.0	5	14.4	43	7.49	1.2	4.5
			Week 17			
Male						
0	15	16.0	46	7.57	0.9	6.4
0.2	15	15.4	45	7.44	0.6	7.3
1.0	15	14.9[g]	43	6.97	0.8	6.3
2.0	15	14.5[g]	43	7.60	0.9	6.3
Female						
0	15	14.9	43	7.14	0.9	4.7
0.2	15	14.9	44	7.05	0.8	4.5
1.0	15	14.5	42[h]	7.26	1.0	5.4
2.0	15	13.8	42[h]	6.78	0.8	5.5

[a] Figures are means for the numbers of rats shown.

[b] Hb = Hemoglobin.

[c] PCV = packed cell volume.

[d] RBC = red blood cells.

[e] Retics = reticulocytes.

[f] WBC = white blood cells.

[g] $P < 0.01$.

[h] $P < 0.05$.

[i] $P < 0.001$.

Source: Adapted from Gray et al., 1977. Reprinted with permission of the publisher.

heart weight, an increase in male brain weight, an increase in female kidney weight, and an increase in ovary weight. However, by the 17th week, no new organs were affected, although there were quantitative changes. An examination of the data in this study reveals that the majority of effects were indicated by the 2-week study, and certainly by

Table 4.27. Relative organ weights of rats fed diets containing 0 to 2.0% di-(2-ethylhexyl)phthalate (DEHP) for 2, 6, or 17 weeks

Sex and dietary level (%)	Number of rats examined	Relative organ weight (g per 100 g of body weight)											
		Brain	Heart	Liver	Spleen	Kidneys	Stomach	Small intestine	Cecum	Adrenals[a]	Gonads[b]	Pituitary	Thyroid[a]
[illegible]													
Male													
0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
1.0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2.0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Female													
0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]
1.0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2.0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]													
Male													
0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
1.0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2.0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Female													
0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]
1.0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2.0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]													
Male													
0	15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
0.2	15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
1.0	15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2.0	15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Female													
0	15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
0.2	15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
1.0	15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2.0	15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[a] Relative weights of these organs are expressed in mg per 100 g of body weight.

[b] Relative weights of the female gonads are expressed in mg per 100 g of body weight.

[c] P < 0.01.

[d] P < 0.001.

[e] P < 0.05.

Source: Adapted from Gray et al., 1977. Reprinted with permission of the publisher.

the 6th week. The only significant change found with 17 weeks of testing was the quantitative degree of toxicity in some organs.

4.3.4 Conclusions

The literature contains many well-designed studies evaluating the predictability of chronic (lifetime) effects from subchronic data. The review papers by Weil and McCollister (1963) and McNamara (1976) presented compilations of data demonstrating that the 90-day or 90-exposure test is a significant predictor of chronic effects. Other authors generally agreed with this conclusion, with many suggesting a duration of 3 to 6 months as sufficient (Barnes and Denz, 1954; Bein, 1963; Boyd, 1968; Davey, 1964; Peck, 1968; World Health Organization Technical Report, 1966). In addition, several primary studies showed that chronic effects are indicated at a statistically significant level by tests of 90 days (Ambrose et al., 1972; Gaunt et al., 1965; Kociba et al., 1976, 1978; Rosenkrantz et al., 1975). Thus, the evidence in the literature supports the conclusion that a 90-day test duration does predict most chronic effects.

There are many comparisons in the literature concerning test durations shorter than 90 days. Generally, these conclude that the shorter tests do indicate toxic effect patterns. However, one must consider that many of the primary studies of short duration produced effects only at the high dose, which reduced the reliability of their conclusions. Papers by Smith (1950) and Weil et al. (1969) concluded that short tests (7 to 14 days) could be sufficient to indicate toxic effects and predict safe dose levels. Two papers by Kast et al. (1975*a*, 1975*b*) indicated that a 1-month duration would adequately predict 6-month effects. Thirty-day durations have also been evaluated and generally recommended as sufficient to indicate toxicity or dose levels (Gray et al., 1977; Hall, Austin, and Fairweather, 1966; Harris et al., 1973; Hodge, Downs, Panner, Smith, Maynard, Clayton, and Rhodes, 1967; Krussye et al., 1977; Misu et al., 1966). However, Hayes (1972) concluded that 30-day durations were insufficient, but in an early paper (Hayes, 1967*b*) did agree that a 90-day duration was a good indicator of chronic effects. Thus, data from primary studies generally support the use of 30-day duration, although there is little concurrence on what toxic effects might be missed. Perhaps in the future, with more information, specific recommendations can be made. In any case, the acceptance of a 90-day test as a significant evaluation of chronic effects can reduce the cost and time factors. This could reduce costs from approximately $255 800 for a complete chronic and subchronic study to $100 500 for a subchronic study design (Gehring, Rowe, and McCollister, 1973).

4.4 ROUTE OF EXPOSURE

4.4.1 Introduction

In subchronic test designs, various routes of administering the chemical to the test animals have been utilized. The routes most often employed are oral and inhalation. Alternate exposure methods such as percutaneous application are used if advantageous for a specific chemical. In 183 subchronic studies taken from the literature, the frequency for employment of each route is as follows: oral, 51%; inhalation, 19%; percutaneous, 2%; intravenous, 8%; intraperitoneal, 8%; subcutaneous, 4%; and others, 8%.

The choice of which route to use should be based on the expected route of human exposure (Benitz, 1970; *Federal Register*, 1978; Food Safety Council, 1978; Ministry of Health and Welfare Canada, 1975; National Academy of Sciences, 1975, 1977; Peck, 1974; World Health Organization, 1978). In addition to the metabolic reasons for using the expected human exposure route, it is easier to extrapolate safe dose levels from test data to the actual human conditions if the same routes are used. However, in some cases it may not be advisable to use only the expected human route. If there are several potential routes of exposure, it may be necessary to test each one to determine the most toxic route. This can be important when the major route of exposure is not the most toxic (U.S. Environmental Protection Agency, 1979). The effect at the site of exposure is another important consideration since the concentration is controlled by the mode of exposure (U.S. Environmental

Protection Agency, 1979; Weisburger, 1975; World Health Organization, 1978). Also, the availability of testing facilities may determine the choice of exposure route, especially for inhalation studies. In most cases, however, there is little or no conflict among these variables, and the route chosen is the expected route of human exposure.

The following section will discuss aspects of individual routes used most frequently in subchronic tests. An additional section will briefly consider the primary literature base for comparison of routes and the validity of extrapolation between routes.

4.4.2 Route Discussions

Oral administration of chemicals is the principal route used in most toxicity experiments. This predominance results from both practical considerations, including ease of application and low cost factors, and experimental requirements (oral exposure represents the expected primary route of human exposure for many substances). In gereral, oral exposures produce quick responses of intermediate toxicity. Hayes (1967a) rates oral exposure as more toxic than dermal applications, while the World Health Organization (1978) rates oral (gavage) as more toxic than dermal but less than inhalation exposures. On a more specific level, oral administration of quickly absorbed chemicals produces more liver toxicity than any other route (Loomis, 1974; World Health Organization, 1978); slowly absorbed chemicals produce more gastrointestinal damage (Ministry of Health and Welfare Canada, 1975).

Oral administration can be performed by various techniques, including mixture in diet (food or drinking water), gavage (gastric intubation), and gelatin capsules or pressed tablets. The relative frequency of use for these techniques in 95 oral application studies surveyed was as follows: gavage, 27%; capsule, 11%; and diet, 60% (89% in food; 11% in water).

The most common technique for oral exposure of chemicals is by mixing the test substance into the diet. One of the advantages of this method is the reduced handling time due to the simplicity of administration. This saves valuable personnel time and disturbs the test animals less (Barnes and Denz, 1954). Diet mixtures also produce a low, prolonged exposure with few concentration peaks (Benitz, 1970). This exposure pattern is more representative of chronic human exposure than other oral routes (Sontag, Page, and Saffiotti, 1976). However, one must be aware of possible shortcomings or hazards involved in the use of diet mixtures. These include: limited duration for stability of the drug in the diet (Benitz, 1970; World Health Organization, 1978); adjustment of the dose with animal maturation to maintain proper concentration levels (Barnes and Denz, 1954; National Academy of Sciences, 1977; World Health Organization, 1978); alteration of nutrient availability or quality in the diet (Barnes and Denz, 1954; Food Safety Council, 1978); unpalatability of the diet mixture to test animals affecting growth rates (Barnes and Denz, 1954); nonhomogeneity of the diet-chemical mixture (Sontag, Page, and Saffiotti, 1976); and measurement of daily food consumption to calculate daily exposures (Barnes and Denz, 1954; Benitz, 1970; Ministry of Health and Welfare Canada, 1975). The choice between diet and gavage or capsule application is often based on such considerations. Use of gavage or capsules eliminates problems of palatability, drug stability, nutrient integrity, and consumption calculations associated with diet administration. However, gavage or capsules tend to produce peaks in substance concentrations, giving an irregular cycle to blood and tissue levels (Benitz, 1970). Also, only small amounts of a compound can be administered by gavage, thus limiting its use to highly toxic substances (Food Safety Council, 1978). Other disadvantages include higher mortality rates and more frequent need to use a solvent to administer the chemical (Sontag, Page, and Saffiotti, 1976).

Often the best choice depends on the chemical being tested. Worden and Harper (1963) and Bein (1963) gave examples of chemicals that are more toxic by feed than by gavage and vice versa. If there is little difference between the techniques, diet is to be preferred because of its simplicity (World Health Organization, 1978). However, either method is acceptable for exposure in tests involving subchronic durations (Ministry of Health and Welfare Canada, 1975; National Academy of Sciences, 1977).

Inhalation exposure is used in studies where the primary human exposure is to be by inhalation or when information on the specific site of entry (lung) damage is desired (Roe, 1968). The total effects of inhalation can rarely be predicted (with a high degree of certainty) from oral or parenteral studies, which necessitates the use of inhalation exposure (Gage, 1970; World Health Organization, 1978). However, unlike oral administration, use of inhalation exposure is neither simple nor inexpensive.

For an adequate study of inhalation toxicity, specially designed inhalation chambers are needed. The various chamber designs and advantages are too numerous to discuss in this document, but Roe (1968), the National Academy of Sciences (1977), and the World Health Organization (1978) provide good reviews of this area. Additional important considerations are the increased cost that this route of exposure entails and the potential bottleneck represented by the limited number of quality facilities available for testing.

One aspect of the test design that must be considered is the difficulty associated with the determination of dose levels in an inhalation study. The dose is usually measured by monitoring the chamber concentration (*c*) of the toxicant and the time (*t*) that the test animal is in the chamber (MacFarland, 1968; Roe, 1968). The dose is expressed as the product (*ct*) of these two variables (Ministry of Health and Welfare Canada, 1975) and is used in lieu of actual dosage levels (National Academy of Sciences, 1977). However, doses given in this manner are difficult, if not impossible, to correlate with oral or injection studies where the dose is expressed as milligrams per kilogram of body weight (Barnes and Denz, 1954; Hayes 1967*a*). Additional problems associated with inhalation dosage are the uncertainties regarding the actual amount of the toxicant inhaled by the test animal, the amount of toxicant that adsorbs onto the chamber walls, and possible variations in the flow rate of the toxicant into the chamber (National Academy of Sciences, 1977; World Health Organization, 1978). The amount of toxicant that enters the test animal is influenced by its ventilation rate, the particle size or vapor pressure of the toxicant, and the behavioral traits of the test species (National Academy of Sciences, 1977; Roe, 1968).

Another factor of importance in the chamber design is the expected schedule of exposure durations. If continuous exposures (22 to 24 h/day, 7 days/week) are to be used, then the chamber must provide all food, water, and other needs of the test animals. If intermittent exposures (6 to 8 h/day, 5 days/week) are used, chambers of a simpler design can be used. These two duration patterns produce different effects. The nonexposure time in an intermittent test design allows the animal to recover and tends to produce a pulsed chemical concentration pattern (World Health Organization, 1978). In contrast, the continuous exposure results in a steady-state pattern of toxin concentrations. The choice of duration schedules should be related to the expected human exposure pattern.

The generation and characterization of aerosols is another complexity involved with inhalation studies. This is of little concern in vapor or gas studies, but in aerosol mist or dust studies the aerosol particles must be evaluated. Specialized equipment is required to generate the aerosol particles, and some control is necessary to ensure uniform particle size and density, at least within a size range that is suitable for biological action (Ministry of Health and Welfare Canada, 1975; National Academy of Sciences, 1977; Roe, 1968). Particle size must also be monitored in the chamber itself to ensure that the exposure levels remain consistent. The deposition sites (nose, trachea, bronchi, or alveoli) and the degree of biological action of the inhaled particles are determined by their solubility in tissue fluids, their reactivity with lung tissue, and their size (Roe, 1968; World Health Organization, 1978). Generally the larger particles, 5 to 10 μm in diameter, are trapped in the upper respiratory tract and nasal cavity (Ministry of Health and Welfare Canada, 1975; World Health Organization, 1978). Particles of less than 5 μm disperse further into the lower respiratory tract, with the depth of penetration increasing with decreasing particle size (World Health Organization, 1978). It has been estimated that approximately 25% of the inhaled particles are immediately expelled, 50% are trapped in the upper respiratory tract, and only 25% reach the alveoli (Morrow et al., 1966).

After inhalation exposure, two types of toxic effects can occur. Systemic effects, not related to the site of entry, are often observed and in most studies represent the major toxic effects (Barnes and Denz, 1954; World Health Organization, 1978). These effects are quite similar to parenteral or intravenous injection effects, at least in qualitative data (Gage, 1970; World Health Organization, 1978). In contrast to oral exposure, the circulatory transport of toxins entering through the lung does not carry them through the liver first. Thus, other organs (e.g., brain, heart, and endocrine glands) are often sites of damage after inhalation exposure (World Health Organization, 1978). Specific site of entry effects (lung damage) can be studied only with inhalation exposure (Roe, 1968). These effects result from both physical impairment of respiratory function and slow metabolic transformation and transport by lung epithelial tissue. Tests to evaluate this specific lung and tracheal damage are reviewed by Roe (1968) and the World Health Organization (1978). These include respiratory function tests, as well as pathological damage evaluations.

A complication in evaluating toxic effects from inhalation is the clearance mechanism. The mucociliary system often transports particles out of the lung. These are either expelled or swallowed. If expelled, the dose levels are not as potent as originally designed. If swallowed, oral exposure results, which produces different types of toxic damage (World Health Organization, 1978). These problems are most often associated with the dust or aerosol studies.

The unique value of inhalation exposure in subchronic test designs is primarily for the assessment of specific lung damage. Because of the high cost, limited facilities, and additional personnel time involved, inhalation exposure should be limited to such assessments and not used for general assessment of systematic toxicity.

Other routes of exposure are infrequently used in subchronic studies, including percutaneous and injection methods. Percutaneous or dermal application is usually performed as a separate study. When percutaneous exposure is used, several factors influence the test design and data interpretation. These include slower activity and lower exposures than with oral exposure, difficulty of measuring absorbed dose, necessity of using a vehicle or solvent in many cases, and ingestion by animals during self-cleaning of doses applied to the skin (Benitz, 1970; Loomis, 1974; Ministry of Health and Welfare Canada, 1975; National Academy of Sciences, 1977).

Parenteral injections are also used occasionally in subchronic tests. These include intradermal, subcutaneous, intramuscular, intravenous, intraperitoneal, and intrathecal (Loomis, 1974). Intravenous administration produces rapid distribution of toxins, with the degree of metabolic transformation dependent upon the location of the used blood vessel in the body. The other injection methods concentrate doses in selected areas with slow diffusion to other tissues (Loomis, 1974). In any injection method, rotation of injection sites is necessary (Benitz, 1970). Although this prevents excessive entry site damage, the use of different target sites complicates the analysis of effects. In most subchronic studies, the use of parenteral administration is not the preferred route, because it is rarely an expected route of human exposure.

4.4.3 Route Comparisons

Few papers in the literature discuss in detail the comparative value of exposure routes. The review articles used in Sect. 4.4.2 contain most of the available information. Primary studies designed to evaluate this area are almost nonexistent. Even within individual chemicals, comparisons are difficult. The use of different species, doses, durations, and parameters of evaluation (pathology, hematology, etc.) all combine to make route comparisons feasible only at a general level. A few brief comparisons within chemicals are included as examples of the difficulties. These are summarized in Table 4.28. Following the chemical comparisons will be a brief discussion of route-to-route extrapolation.

4.4.3.1 Arsenic. Bencko and Symon (1969) studied tissue levels of arsenic in hairless mice following administration of white arsenic (As_2O_3) in drinking water. Continuous exposure to 50 mg or 250 mg of

Table 4.28. Summary of chemical examples tested for toxicity by various routes of administration

Chemical	Route	Species	Dose	Duration	Effects noted by author	Reference
Arsenic	Oral	Mouse	[illegible] to 17 mg/kg/day	42 days	Transient accumulation in skin and liver; weight loss	Bencko and Symon, 1969
	Inhalation	Mouse	179.4 µg As per m^3	6 h/day, 5 day/week, 6 weeks	Transient accumulation in skin, liver, and kidney	Bencko and Symon, 1970
Beryllium salts	Oral	Guinea pig	[illegible] to 30 mg/kg/day	12 to 24 weeks	No adverse effects; low tissue levels	Hyslop, 1943
Insoluble salts	Inhalation	Guinea pig	128.4 and 23[illegible] mg/m^3	30 to 40 min/day, 5 days/week, 1 day to 13 weeks	No adverse effects; low tissue levels	Hyslop, 1943
Soluble compounds	Inhalation	Guinea pig	17 and 43 mg/m^3	30 to 60 min/day, 5 days/week, 1 and 10 doses	Severe symptoms; low tissue levels	Hyslop, 1943
Beryllium chloride	Intraperitoneal	Guinea pig	0.1 g/day	4 days	100% mortality	Hyslop, 1943
Beryllium nitrate	Intraperitoneal	Guinea pig	0.1 g/day	4 days	100% mortality	Hyslop, 1943
Beryllium sulfate	Intraperitoneal	Guinea pig	0.1 g/day	4 days	100% mortality	Hyslop, 1943
Beryllium oxyfluoride	Intraperitoneal	Guinea pig	0.1 g/day	4 days	100% mortality	Hyslop, 1943
Beryllium oxide	Intraperitoneal	Guinea pig	0.1 g/day	4 months	No mortality	Hyslop, 1943
Beryllium phosphate	Intraperitoneal	Guinea pig	0.1 g/day	4 months	No mortality	Hyslop, 1943
Beryllium	Oral	Rat	5.4 and 54.0 µg/day	6, 12, 18, 24 months	Decreased body weight; accumulation in gastrointestinal tract; skeleton, blood, liver. No hepatic cell destruction.	Reeves, 1965
Beryllium sulfate	Inhalation	Rat	34.25 µg Be per m^3	7 h/day, 5 days/week, 72 weeks	Inflammatory response; lung tumors	Reeves, Deitch, and Vorwald, 1967
Beryllium fluoride	Inhalation	Rabbit, dog	1.2 mg/m^3	6 h/day, 5 days/week, 6 to 13 weeks	Macrocytic anemia	Stokinger and Stroud, 1951
Beryllium sulfate	Inhalation	Dog, rat	4 mg/m^3	6 h/day, 5 days/week, 6 to 23 weeks	Macrocytic anemia	Stokinger and Stroud, 1951
Beryllium oxide	Inhalation	Rabbit	30 mg/m^3	6 h/day, 5 days/week, 6 to 23 weeks	Macrocytic anemia	Stokinger and Stroud, 1951
Carbon tetrachloride	Inhalation	Rat	Not given	3 to 5 min, 3 times per day on alternate days to 35 days	Cirrhosis of liver	Reddy, Krishnamurthy, and Bhaskar, 1962
	Inhalation	Rat	10, 50, 100 ppm/day	5 h/day, 6 to 8 weeks	Elevated liver triglycerides, SGOT,[a] SGPT[b] unaffected	Shimizu, Nagase, and Kawai, 1973

Table [illegible] (continued)

Chemical	Route	Species	Dose	Duration	Effects noted by author	Reference
	Oral	Rat	[illegible] per 100 g	1 day (acute)	(Single dose) elevated SGOT,[b] SGPT	Shimizu, Nagase, and Kawai, 1973
	Oral	Rat	100 to [illegible] ppm/day	6 weeks	Triglyceride accumulation in liver	Alumot et al., 1976
	Oral	Rat	0.2 ml/kg/day	[illegible] days/week, 7 weeks	Increased liver fat	Friedman, Sage, and Blendermann, 1970
	Subcutaneous	Rat	0.2 ml/100 g	[illegible] dose per [illegible] days (to 90 days)	Cirrhosis	Reddy, Krishnamurthy, and Bhaskar, 1962
	Subcutaneous	Rat	1.3 ml/kg/day	[illegible] days/week, 12 weeks	Cholangiofibrosis	Reuber and Glover, 1967a
	Subcutaneous	Rat	1.3 ml/kg/day	[illegible] days/week, 12 weeks	Hepatic vein thrombosis	Reuber and Glover, 1967b
	Subcutaneous	Rat	1.3 ml/kg/day	[illegible] days/week, 12 weeks	Neoplasia	Reuber and Glover, 1967c
	Subcutaneous	Rat	1.3 ml/kg/day	[illegible] days/week, 12 weeks	Cirrhosis	Reuber and Glover, 1968
	Intraperitoneal	Rat	[illegible] ml/kg (as [illegible])	[illegible] injections for 110 days	Retardation of growth; elevated SGPT; hepatic necrosis (transient); inhibition of normal mineralization of tooth dentine	Hals, Bjorlin, and Jacobsen, 1973
Dimethyl terephthalate	Oral	Rat	0.25, [illegible] ppm/day	96 days	Decreased body weight	Krasavage, Yanno, and Terhaar, 1973
	Inhalation	Rat	16.5 and 86.4 mg/m³	4 h/day, 5 days/week, 58 exposures	No adverse effects	Krasavage, Yanno, and Terhaar, 1973
Fenoterol HBr	Oral	Mouse, rat	[illegible], [illegible], 1000 mg/kg/day (rat) [illegible], [illegible], [illegible] mg/kg/day (mouse)	[illegible] days [illegible] days	Enlarged salivary glands; enlarged hearts. Mortality: rats – 64% male, 24% female; mouse – [illegible] male and [illegible] female	Kast et al., 1975a
	Inhalation	Rat	[illegible] to [illegible] mg/kg/day	[illegible] days	No adverse effects; [illegible] mortality	Kast et al., 1975a
	Intravenous	Rabbit	[illegible] and [illegible] mg/kg/day	[illegible] days	Enlarged salivary glands; enlarged hearts; 6% mortality	Kast et al., 1975a
2-Methyl-4-chlorophenoxy acetic acid (MCPA)	Dermal	Rabbit	0.5, 1.0, 2.0 g/kg/day	3 weeks	Transient growth retardation; erythema; decreased leukocytes; hyperplasia, hyperkeratosis, and loss of elasticity of skin 100% and 75% mortality in high dose groups	Verschuuren, Kroes, and Tonkelaar, 1975

[illegible]

Chemical	Route	Species	Dose	Duration	Effects noted in Animals	Reference
	Oral	[illegible]	[illegible]	[illegible]	Growth retardation Increased kidney weight [illegible] Decreased [illegible] content	[illegible], [illegible], and [illegible], [illegible]
2-Methyl-chlorophenoxy-propionic acid (MCPP)	Dermal	[illegible]	[illegible]	[illegible]	[illegible]	[illegible], and [illegible], [illegible]
	Oral	[illegible]	[illegible]	[illegible]	[illegible]	[illegible], and [illegible], [illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible], Smith, and Nelson, [illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	No adverse effect	[illegible], Smith, and Nelson, [illegible]
	Oral	[illegible]	[illegible]	[illegible]	No adverse effect	[illegible], Smith, and Nelson, [illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	Litter weight [illegible]	[illegible], Smith, and Nelson, [illegible]
	Oral	[illegible]	[illegible]	[illegible]	Litter weight [illegible]	[illegible], Smith, and Nelson, [illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	No adverse effect	[illegible], Smith, and Nelson, [illegible]
[illegible]	Oral	[illegible]	[illegible]	[illegible]	[illegible]	[illegible] et al., 1975
	[illegible]		[illegible]	[illegible]		

SGOT - serum glutamic oxaloacetic transaminase.

SGPT - serum glutamic pyruvic transaminase.

arsenic per liter (10 to 15 mg/kg per day) for 32 days produced levels of the chemical in the skin and liver that peaked at 16 days, then decreased significantly by 32 days.

In an inhalation experiment (Bencko and Symon, 1970), hairless mice were exposed in a dust chamber to As_2O_3 dried onto fly ash at a mean concentration of 179.4 μg arsenic per m^3 for 5 days per week, 6 h daily, for 6 weeks. Concentrations of arsenic in the liver, kidney, and skin were measured after 1, 2, 4, and 6 weeks of exposure. Arsenic levels increased rapidly in the liver and kidney up to 2 weeks, then decreased sharply during the third and fourth weeks. Skin values peaked similarly as a result of direct exposure in the dust chamber, but decreased more gradually. The authors recognized that effects of inhaled particles cannot be distinguished from effects of particles ingested as a result of mucociliary clearance or licked from the skin during grooming, and they concluded that the course of arsenic accumulation is similar following oral administration and inhalation. The drop in arsenic accumulation in tissues after the initial peak was subsequently shown to be caused by a stimulation of the excretory mechanism, which increased the excretory rate of the animals (Bencko, Dvorak, and Symon, 1973).

4.4.3.2 Beryllium In an extensive test for beryllium toxicity (Hyslop, 1943), guinea pigs ingested up to 40 mg per kg of body weight per day of various beryllium salts and exhibited no adverse effects after 11 to 29 weeks of exposure. The total amounts of beryllium retained in body tissues were small in comparison to the quantities ingested. Inhalation of insoluble beryllium carbonate (188.9 mg/m^3 or 233 mg/m^3) 30 to 40 min/day, 6 days/week for 1 day to 15 weeks, failed to produce toxic effects in guinea pigs. However, inhalation of soluble compounds induced severe symptoms in a short time: 27 mg/m^3 of beryllium oxyfluoride and 43 mg/m^3 beryllium sulfate induced 67% mortality after 10 days and 1 day of exposure, respectively. Distribution of the chemical in tissues remained remarkably low following inhalation of both soluble and insoluble beryllium salts. Intraperitoneal injections of 0.1 g of beryllium chloride, beryllium nitrate, beryllium sulfate, or beryllium oxyfluoride produced 100% mortality in guinea pigs after 4 days; beryllium oxide and phosphate were not lethal after 4 months.

Other studies of beryllium toxicity are summarized in Table 4.[illegible]. These are of little value for route comparisons because of dose, duration, and species differences.

4.4.3.3 Carbon Tetrachloride (CCl_4) Reddy, Krishnamurthy, and Bhaskar (1962) exposed albino Wistar rats to carbon tetrachloride (CCl_4) vapors for 3 to 5 h, three times per day on alternate days for 35 days. Inhalation of the vapors caused cirrhosis of the liver in 8 of 9 males and 5 of 15 females tested. Eight of the 15 females developed patchy fibrosis of the liver. Inhalation of 10, 50, or 100 ppm CCl_4 per day, 3 h/day for 6 to 8 weeks resulted in temporarily elevated triglyceride levels in the livers of male Sprague-Dawley rats, but no progressive or cumulative changes were observed (Shimuzu, Nagase, and Kawai, 1973). Transaminase activity was not affected by inhalation exposure, although a single oral dose of CCl_4 produced increased SGOT and SGPT values.

Elevated levels of liver fat content have been observed in rats following oral administration of CCl_4. After 6 weeks of feeding fumigated mash containing a daily dose of 1% of the LD_{50} (5 g per kg of body weight), triglyceride accumulation in the liver was found to be the most sensitive sign of chronic CCl_4 poisoning (Alumot et al., 1976). Oral intubation of 0.2 mL CCl_4 per kg of body weight three times a week for 7 weeks also caused an increase in the liver fat content of rats (Friedman, Sage, and Blendermann, 1970).

Subcutaneous injection of 0.1 mL per 100 g of body weight every 3 days for 90 days produced cirrhosis in 9 of 9 male rats and in 6 of 15 female rats (Reddy, Krishnamurthy, and Bhaskar, 1962). Five females developed patchy fibrosis of the liver, and four females did not develop liver lesions. The authors concluded that the frequency of CCl_4-induced cirrhosis was the same following inhalation (described previously) and subcutaneous exposure. In a series of separate studies in which CCl_4 was administered subcutaneously to Buffalo strain male and female rats, effects of the chemical were manifested as a function of age and sex of the animals (Reuber and Glover, 1967*a*, 1967*b*, 1967*c*, 1968).

Intraperitoneal injection of 0.06 mL of CCl_4 per 100 kg of body weight (up to seven injections during 119 days) into albino rats induced an inhibition of weight gain, elevated SGPT values, and hepatic necrosis, which then regenerated (Hals, Bjorling, and Jacobsen, 1972). Concomitantly, inhibition of normal mineralization of the dentine layer of rat incisors was observed.

These ten studies on carbon tetrachloride, although using the same species, contain wide variations in dose and duration. Also, the use of different age groups in some studies further complicates the comparisons. Thus, comparisons of routes using these data would be difficult.

4.4.3.4 Dimethyl Terephthalate (DMT) — DMT, shown previously to be of a low order of toxicity when administered intragastrically, intraperitoneally, and subcutaneously to rats and mice (Slyusar and Cherkasov, 1964, and Prusakov, 1966, as cited in Krasavage, Yanno, and Terhaar, 1973), was further evaluated in various acute studies and in subchronic feeding and inhalation studies by Krasavage, Yanno, and Terhaar (1973). Neither feeding nor inhalation caused mortality. The toxicological effect seen in either study was reduced weight gain in high dose animals of the feeding study. Although this study was well designed for a comparison of routes, the lack of toxic effects precludes comparisons.

4.4.3.5 Fenoterol HBr — Kast et al. (1975a) tested the beta-sympathomimetic, fenoterol HBr, for toxic properties. Subacute exposures (oral, intravenous, and inhalation) continued for 1 month. Inhalation produced no symptoms in rats that could be related to fenoterol. However, after oral administration enlarged salivary glands and enlarged hearts (with some myocardial scarring) were evident in both rats and mice. Less than 1% of the rats in the inhalation experiment died during the exposure period, while oral administration of high doses produced mortality rates of 64% for male rats, 24% for female rats, and 3% for male and female mice. Rabbits receiving the drug intravenously developed enlarged salivary glands and enlarged hearts. Six percent of the rabbits died during the third week of the trial. In this study, the durations were all comparable, but the use of various species and different doses reduced the reliability of the comparisons.

4.4.3.6 2-Methyl-4-Chlorophenoxy Acetic Acid (MCPA) — In a subacute dermal study, doses of 0, 0.5, 1.0, and 2.0 g MCPA per kg of body weight were applied to the skin of chinchilla rabbits in an aqueous paste (Verschuuren, Kroes, and Tonkelaar, 1975). Four of four rabbits treated with 2 g MCPA and three of four treated with 1 g MCPA died during the treatment period, possibly as the result of dysbacteria (an illness often seen in rabbits of that institute). Growth retardation was observed in animals of the 0.5-g/kg group. However, growth rates improved during the recovery period. Other major changes noted and attributed to dermal application of MCPA were erythema; a decrease in the number of lymphocytes in the 0.5-g/kg group; and hyperplasia, hyperkeratosis, and loss of elasticity of the skin.

In the subchronic oral study, male and female rats received 0, 50, 400, or 3200 ppm MCPA in their food for 90 days (Verschuuren, Kroes, and Tonkelaar, 1975). During the first week's feeding of 3200 ppm the rats exhibited unhealthy fur and cold extremities. There was no mortality attributed to dosage. Decreased food consumption, decreased body weight gain, and increased kidney weights were characteristic of the 3200-ppm group. There was also an increase in erythrocyte size and a proportional increase in hemoglobin content in rats of the high dose group.

4.4.3.7 2-Methyl-4-Chlorophenoxy Propionic Acid (MCPP) — In a subacute dermal study, doses of 0, 0.5, 1.0, and 2.0 g MCPP per kg of body weight were applied to the skin of chinchilla rabbits in an aqueous paste (Verschuuren, Kroes, and Tonkelaar, 1975). One rabbit of the 1.0-g/kg group and one rabbit of the 2.0-g/kg group died. Growth retardation was seen in all groups, but some recovery was observed during the 2 weeks after termination of treatment. Erythema was observed and was dose related. The skin lost its elasticity, but reverted to normal condition during the recovery period. Organ weights of the treated animals were no different from those of the controls.

In a subchronic oral study, male and female rats received 0, 50, 400, or 3200 ppm MCPP in their food for 90 days (Verschuuren, Kroes, and Tonkelaar, 1975). Rats of both sexes treated with 3200 ppm had unhealthy fur, decreased hemoglobin content, decreased erythrocyte counts, and increased alkaline phosphatase activity; hematocrit and leucocyte values were decreased in males only. Some of the changes observed also occurred with 400 ppm. Increased kidney weights were seen in rats at levels of 3200 and 400 ppm, and depression of ovary and prostate weights was seen with 3200-ppm feedings.

These two studies of MCPA and MCPP are typical of many primary studies in the literature. Although two routes of exposure are used, they differ in species, duration, and dose. This makes them of minimal value for comparisons.

4.4.3.8 Nefopam Hydrochloride — Nefopam, a compound with nonnarcotic analgesic activity, was tested for subacute toxic properties by Case, Smith, and Nelson (1975). Carworth CFN rats were administered the drug by three routes: intraperitoneally, in daily doses of 0, 2, and 10 mg/kg (5 days per week for 4 weeks); intramuscularly, in daily doses of 0, 1, and 2 mg/kg (7 days per week for 2 weeks); and orally, in the diet, in daily doses of 0, 20, 40, and 80 mg/kg for 4 weeks. Increased liver weights were observed in female rats receiving 10 mg/kg of the drug intraperitoneally (approximately 30% of the intravenous LD_{50}). No indications of toxicity were seen in any of the other groups receiving up to 2 mg/kg per day intramuscularly (approximately 3.5% of the intramuscular LD_{50}) or up to 80 mg/kg per day orally (approximately 65% of the oral LD_{50}).

Mongrel dogs showed only slight weight loss after daily intravenous administration of 5 mg/kg (approximately 25% of the intravenous LD_{50}) and after daily oral administration of 4 and 40 to 80 mg/kg (25% to 50% of the oral LD_{50}) of nefopam. No toxic effects were observed following daily intramuscular injections of up to 3 mg/kg of body weight (approximately 10% of the intramuscular LD_{50}) of the drug.

Despite using three routes of exposure in both the rat and the dog, the study by Case, Smith, and Nelson (1975) still differs in dose and duration so that comparisons would be difficult.

4.4.3.9 Δ^9-Tetrahydrocannabinol (Δ^9-THC) — Thompson et al. (1974) described the toxic effects of Δ^9-tetrahydrocannabinol, the active ingredient in marijuana, when adminstered intravenously or orally to primates for 28 days. In a preliminary acute study rhesus monkeys survived up to 9000 mg/kg orally, while 128 mg/kg intravenously produced 100% mortality (two of two). The subacute study consisted of male and female monkeys, either treated with oral doses of 0, 50, 250, or 500 mg/kg per day or injected intravenously with 0, 5, 15, or 45 mg/kg per day. During oral administration, two male monkeys treated with 500 mg/kg per day and one monkey treated with 50 mg/kg became moribund and were sacrificed on days 10, 14, and 16, respectively. Deaths in the intravenous trial caused by acute hemorrhagic pneumonia occurred only in the 45 mg/kg per day group at days 8 and 19. Table 4.29 summarizes the main toxic effects observed in intravenously or orally treated monkeys.

Table 4.29. Main toxic effects produced by oral or intravenous administration of Δ^9-tetrahydrocannabinol[a]

	Routes of administration	
	Oral	Intravenous
Organ size		
Thymus	Atrophic	Atrophic
Pancreas	Atrophic	
Testes	Atrophic	
Adrenal	Enlarged	
Liver	Enlarged	Enlarged
Body weight	Decreased	
Clinical effect		
Leukocytosis	Yes	Yes
Myeloid hyperplasia	Yes	
Anemia	No	Yes
Bromosulfophthalein retention	No	Yes
Electrolyte balance	Altered	
Proteinuria		Yes
Bradypnea	Yes	Yes
Anorexia	Yes	Yes
Ulcerative colitis	Yes	
Other effects		
Behavioral changes	Yes	Yes
Development of tolerance	Yes	Yes

[a] The authors concluded that pathologic changes in deceased monkeys were associated with route of administration.

Source: Adapted from Thompson et al., 1974.

This study has differences in dose levels that make the conclusions of the authors questionable.

The preceding examples are typical of within-chemical comparisons. Almost every comparison was complicated by test design variables. Any conclusions that could be drawn from these would be tenuous at best. However, this is unfortunately the current status of information on this subject. A computer and manual search of over 30 000 titles and abstracts produced no studies specifically designed to compare exposure routes for subchronic toxicity tests. Without sufficient literature data, an evaluation of the route-to-route extrapolations is limited to a general discussion.

The value of extrapolating data from one route to another is limited. The oral route of exposure has been sufficiently developed to the point that it is easy to perform, requires a minimum of specialized equipment, and produces good toxicity data. Thus, there is little need to extrapolate data from other routes to determine oral toxicity for most chemicals. Injections are rarely primary routes of exposure and are relatively easy to perform for durations of a subchronic nature. Extrapolation from another route is not needed here.

Dermal and inhalation studies are the principal routes for which extrapolation might prove useful. Difficulty in calculating exact dosages taken in by test animals applies to both routes. Also the anatomy and physiology of the entry sites for these routes differ between the test species and man (especially for inhalation), thus affecting the predictive value of data from these two routes. For inhalation, the additional problem of special equipment requirements limits the number of studies that can be performed. Extrapolation might be useful in these routes, if the substituted methods solve these problems. For example, an intravenous injection could be used to replace an inhalation evaluation (at least for systemic toxicity) thus reducing the problem of limited facilities. Dosages could also be more stringently controlled. Another alternative route could be the oral route. This would require previous testing to ascertain if any significant metabolic transformations by the gastrointestinal tract occur for that chemical. Of course, these extrapolations would not provide information on toxicity at the site of entry. These data would have to be obtained from preliminary studies (acute or subacute).

Before any of these route extrapolations can be employed, much laboratory research needs to be performed. Specific comparisons over a wide range of chemicals need to be carried out so that the background data would be available. A basis for extrapolation should be sought in pharmacokinetic and metabolism studies that could justify the quantitative and qualitative validity from one route to the other. This research would help to define the types of toxicants and the aspects of each route, for which extrapolation would not be useful.

4.4.4 Conclusions

In selecting the route of exposure for subchronic toxicity tests, the route by which human exposure is most probable is the preferred choice. The routes used in most subchronic testing are oral and inhalation exposures. Oral exposure can be by diet mixtures, gavage, or capsule, all of which are relatively simple and effective. Inhalation exposure requires special equipment, which results in higher costs per test and acts as the limiting factor in determining the number of tests that can be performed. Occasional use is made of percutaneous and intraperitoneal exposures, depending on the chemical, its metabolic pattern, and the expected human exposures.

In some cases, extrapolation of data from one route to determine potential toxicity by another route may be feasible. Some substitution for dermal or inhalation studies could theoretically occur by using data from intravenous or oral studies. However, the literature is currently insufficient to evaluate this area. The lack of data in the literature stresses the need for research on route-to-route comparisons and extrapolations.

4.5 PATHOLOGY

4.5.1 Introduction

Gross and microscopic pathology examinations are currently an integral part of most toxicity tests. Particularly for the subchronic evaluations, extensive organ and tissue examination is required or recommended. The EPA guideline for pesticide evaluations (*Federal Register*, 1978) is a good example of this, recommending for oral administration the microscopic examination of approximately 30 organs or tissues from all animals at the control and high doses. Additionally, any lesions on other organs revealed by gross examination require a histopathological follow-up. For intermediate and low doses, the tissues to be examined depend on the toxic effect, target organ, and lesions found in the gross examination or in the high-dose histology procedures. Table 4.30 gives a typical list of tissues to be examined (Peck, 1968).

Table 4.30. Postmortem studies

Gross

External lesions
Tumors
Abdominal contents
Pelvic contents
Thoracic contents
Cervical tissues
Oral tissues
Voluntary muscle
Bones
Brain
Spinal cord

Organ weights

Histomorphology

Thymus
Lymph node
Thyroid
Parathyroid
Adrenal
Pancreas
Liver
Gallbladder
Kidney
Urinary bladder
Ovary
Uterus
Testes
Prostate
Seminal vesicle
Mammary gland
Heart
Large and small arteries
Lung
Stomach
Duodenum
Jejunum
Ileum
Colon
Bone (with marrow)
Marrow smears
Brain
Pituitary
Voluntary muscle
Eyes
Other tissues as indicated by gross observations and by drug activity

Source: Adapted from Peck, 1968 – An Appraisal of Drug safety Evaluation in Animals and the Extrapolation of Results to Man. In: Importance of Fundamental Principles in Drug Evaluation, D. H. Tedeschi and R. E. Tedeschi, eds. Raven Press, New York. pp. 450-471. Reprinted with permission of the publisher.

Besides the general appearance and microscopic condition of the organs, the organ weight change is also monitored during and at the end of the exposure period. An extensive, detailed protocol for organ examination, removal, fixation, and interpretation is given by Prieur et al. (1973). The value of such extensive examinations is debatable, however, with evidence and opinions supporting both the pro and con arguments. The purpose of this section of the literature review is to give an overview of opinions concerning the use of pathology and the relative predictive efficiency for each organ.

4.5.2 Use of Pathology

In some earlier short-term tests, microscopic examination was not performed because either the exposure periods were considered too short to affect organ tissues (Bratton, 1945) or the ability of the histopathologic examination to add quantitative information to the other observations was doubted (Smyth and Carpenter, 1948). The significance of histopathology was considered to increase somewhat when the results of the short test were to be a preliminary step for a 2-year study (Smyth and Carpenter, 1948). Smith (1950) felt that histopathology could also be useful if a series of compounds were being compared or ranked for relative toxicities. Even in these cases, the tissues to be examined were few, generally restricted to the liver, kidney, heart, and any lesion-bearing organs. This trend toward reduced histopathology in earlier short-term tests was continued by Smyth, Carpenter, and Weil (1951) in their development of the range-finding test. As shown in Table 4.31, these researchers found no material that caused microscopic tissue damage as the first effect (at the lowest dose). They did find that organ weight could be a significant early indicator of toxic effects, particularly weight changes in the liver and kidney.

Barnes and Denz (1954), in a general review of chronic toxicity, also expressed reservations about the value of pathology, particularly histopathology. They identified some of the sources of variation in pathological results as: the subjective nature of the examination; the occurrence of natural disease; and the problems of age changes in the test species. They saw little value in routine sectioning and histo-

Table 4.31. Range-finding data on subacute oral toxicity[a]

Material studied	Maximum dose (g/kg)	Minimum dose (g/kg)	Least daily dose causing symptom (g/kg)					Maximum dose having no effect (g/kg)	Single-dose oral LD_{50}[c] (g/kg)
			Reduced growth	Reduced appetite	Altered liver or kidney weight	Microscopic lesions[b]	Death		
Acrolein[d]	0.0013	0.00017		0.0013	0.003			0.00017	0.046 (0.039-0.056)
Aldol	1.58	0.026	1.58					0.41	2.18 (2.00-2.38)
Allyl alcohol[d]	0.0097	0.0013		0.0097		0.0097	0.0097	0.0050	0.064 (0.056-0.074)
1,3-Butanediol[f]	5.80	0.32						5.80[f]	22.8 (21.8-23.9)
"Cellosolve" (2-ethoxyethanol)[d,e]	1.89	0.052	0.74	0.74	0.74	0.74	1.89	0.21	3.00 (2.51-3.59)
Dichloral urea	3.33	0.012	0.18	3.33	0.18	3.33	3.33	0.046	32 RF
2,4-Dichlorophenoxy ethyl sulfate, sodium	0.66	0.011	0.66		0.20		0.66	0.041	1.41 RF
Diethanolamine (2,2'-iminodiethanol)[e]	0.68	0.0051		0.68	0.090	0.17	0.17	0.020	1.82 (1.66-2.00)
Di(2-ethylhexyl) adipate[e]	4.74	0.16	2.92	2.92	2.92	2.92	4.74	0.61	9.11 (7.28-11.4)
Di(2-ethylhexyl) tetrahydrophthalate[e]	2.67	0.053	2.67		0.69			0.19	114 RF
2-Dimethylaminoethanol (dimethylethanolamine)[e]	0.89	0.043			0.89			0.24	2.34 (2.26-2.42)
2,4-Dimethyl-2-methylene-1,2,4-thiodiazolidine thione	0.10	0.0023	0.10	0.10	0.039			0.012	0.50 (0.48-0.52)
Dimethyl methylene diphenol-p,p'[e]	0.52	0.002						0.52[f]	4.04 (3.71-4.38)
Dimethyl methylene diphenol-p,p', diglycidyl ether	0.16	0.016						0.16[f]	8.14 (7.23-9.16)
Ethylene chlorhydrin (2-chloroethanol)[e]	0.042	0.0024			0.012			0.0024	0.089 (0.067-0.117)
Ethylenediamine[d,e]	0.31	0.036	0.15	0.12	0.31	0.12	0.12	0.036	1.16 (0.98-1.37)
2-Ethyl-1,3-hexanediol[e]	0.70	0.20	0.70					0.48	2.71 (2.52-2.91)
2-Ethylhexanimido (diethyl-2-ethyl hexanoate)	1.53	0.18			1.53			0.44	40 RF
Glycidyl sorbate dimer	0.27	0.012	0.27		0.012	0.27	0.27	0.012[g]	5.75 (5.28-6.25)
2-Methyl-2,4-pentanediol	0.11	0.041						0.11[f]	4.76 (4.27-5.30)
Monoethanolamine (2-aminoethanol)[e]	2.67[f]	0.16			0.64	1.28	1.28	0.32	2.74 (2.39-3.15)
Monoisopropanolamine[e]	2.22	0.14			2.22			0.60	4.26 (3.88-4.67)
Tetrabutyl thiodisuccinate[e]	10.3	0.26	10.3		1.06	10.3	10.3	0.26	100 RF
1,2,3,4-Tetrathia-6,9-diazecane-5,10-dithione	0.35	0.04	0.35		0.17			0.06	8.53 (6.12-11.9)
Triethanolamine[e]	2.61	0.0050	1.27		0.17	0.73	0.73	0.08	9.11 (8.45-9.82)

[a] Materials administered in the food to groups of ten rats for 30 days on each dose level unless noted otherwise.

[b] The dose producing microscopic lesions in liver, kidney, spleen or testis of any rat.

[c] When LD_{50} was derived from an advanced test, the range of 1.96 times the standard deviation is shown in parentheses. The letters "RF" refer to data derived from a range-finding test.

[d] The dose was administered in the drinking water. In this case appetite is judged on the basis of milliliters of water consumed.

[e] Doses administered for 90 days.

[f] The dose shown is the maximum that was administered, and it caused no symptoms.

[g] The dose shown is the minimum administered, and it caused some symptoms.

Source: Adapted from Smyth, Carpenter, and Weil, 1951. Copyright 1951, American Medical Association. Reprinted with permission of the publisher.

logical examination of 20 major organs and suggested that a thorough gross examination would suffice. They also doubted the value of organ weight changes as indicators of toxicity. Barnes and Denz cited Cameron (1952) concerning the increase in organ weights caused by hypertrophy without true damage to the tissues. They suggested that organ weights be used as toxicity indicators only if histology also shows damage.

Zbinden (1963) also questioned the practice of examining all tissues histologically. He felt that to reduce the extensive technical work histology should be done only on high-dose animals and 25% to 50% of the control animals. Further examination would be limited to organs showing changes. In addition, Zbinden recommended a grading system that ranks histopathological findings, thus increasing the accuracy for initial or subtle effects. The role of organ weight changes is secondary when autopsy and histology are done carefully.

In contrast to these earlier authors, more emphasis is placed on organ weight changes than on histopathology as the predominant indicator of pathological damage by Weil and McCollister (1963) in their evaluation of subchronic and chronic tests. As shown in Table 4.32, out of 33 short-term experiments only twice was histopathology the sole toxic indicator at the lowest dose. However, organ weight change was the sole low-dose toxicity indicator seven times. The same trend is shown for 2-year studies, indicating that histopathology is a poor short-term indicator of chronic toxicity. Specifically, among the organs examined only the liver, kidney, testes, and spleen showed weight changes indicating histologic damage. The weight changes of the liver and the kidney increased their value as toxicity predictors in the longer 2-year studies. Based on these findings, Weil and McCollister recommended that for determination of lowest dose level of effect, the liver and kidney should be examined for weight changes and histological damage. Although not as useful as organ weight, histology of the liver and kidney did increase the predictive efficiency. These factors, plus body weight gain, would serve to adequately indicate toxicity.

In a later paper, Weil et al. (1969) again commented on the value of histopathology. In comparing 90-day, 1-week, and single exposure tests, they used primarily the histology and weight changes of the

Table 4.32. Summary of observations of effects detected in short-term and two-year oral studies

(At lowest dosage level in which any effect was detected)

Criterion of effect	Short-term				Two-year				Number this criterion affected at next higher dosage level[c]	
	Number of studies in which this criterion was followed	Number of pertinent studies[a]	Number in which this criterion was affected	Number in which it was the sole effect	Number of studies in which this criterion was followed	Number of pertinent studies[a]	Number in which this criterion was affected	Number in which it was the sole effect	Short-term	Two-year
Mortality	13	27	1	0	13	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Food intake	10	26	2	0	29	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Body weight	13	27	16	4	13	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Organ weights										
Liver	27	22	8	4	30	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Kidney	27	22	1	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Heart	13	13	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Spleen	17	12	4	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Testes	16	13	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Lung	15	12	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Brain	3	7	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Thyroid	10	10	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Stomach	1	10	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Adrenal	10	8	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Gross pathology	13	[illegible]	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Micropathology										
Liver	30	24	8	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Kidney	30	25	7	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Heart	25	13	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Spleen	21	16	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Testes	21	13	1	1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Lung	20	16	1	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Adrenal	16	12	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Pancreas	16	12	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Bone marrow	3	0	0	0	11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Voluntary muscle	3	12	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Stomach	3	7	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Intestine	3	8	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Bladder	2	[illegible]	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Brain	3	0	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Sciatic nerve	2	0	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Table 4.32 (continued)

Criterion of effect	Short-term: Number of studies in which this criterion was followed	Short-term: Number of pertinent studies[a]	Short-term: Number in which this criterion was affected	Short-term: Number in which it was the sole effect	Two-year: Number of studies in which this criterion was followed	Two-year: Number of pertinent studies[a]	Two-year: Number in which this criterion was affected	Two-year: Number in which it was the sole effect	Number this criterion affected at next higher dosage level[b]: Short-term	Number this criterion affected at next higher dosage level[b]: Two-year
Hematology	19	16	1	0	31	24	0	0	0	0
Blood urea nitrogen	10	7	0	0	6	1	0	0	0	0
Clinical urine analysis	1	1	0	0	7	5	0	0	0	0
Central nervous system	1	1	1	1	0	0	0	0	0	0
Neoplasm	33	27	0	0	33	25	0	9	0	0
Fertility	0	0	0	0	5	3	1	0	0	0

[a] Total number of studies in which this criterion was followed minus the studies in which none of the criteria were significantly altered at any dosage level.

[b] Number of times that effects were demonstrated by the criteria when they were not affected at the next lower dosage level.

[c] Highest level fed was the only one affected.

Source: Adapted from Weil and McCollister, 1963. Reprinted with permission of the *Journal of Agricultural and Food Chemistry*. Copyright by the American Chemical Society.

kidney and liver as indicators of toxic dosages. However, for the 90-day studies, they examined additional organs for toxic changes. The results from the additional organs were less sensitive than the other criteria selected, and they again suggested that histopathology be limited to certain organs such as the liver, kidney, or spleen.

Peck (1968), in his discussion of pathology in drug evaluations, questioned the value of organ weight changes without a histopathological examination as a follow-up. He stated that, when organ weight changes are reflective of toxic effect, histopathology will also show results. But if histopathology is negative, the organ weight change is most likely not due to the toxic action of the drug. Therefore, to be sure that the effect indicated by organ weight change is a result of the tested substance, a histopathology examination is necessary. Peck also stated that histopathology is useful in determining the significance of spontaneous species-specific lesions. If the commonly occurring lesions for a species are altered in time of appearance, number, character, or severity, this could be due to the activity of the drug. However, as a caution to the use of histopathology, Peck stressed the difficulty in interpreting results (particularly for electron microscopy) and in extrapolating results from the test species to man.

Benitz (1970), in his review of chronic toxicity, also stressed the need for a complete histopathological examination. He emphasized that to achieve the maximum results from pathology techniques, a thorough gross necropsy is the critical starting procedure. If the organs are excised improperly the extent of later microscopic examination can be irrelevant. This includes, of course, all organs, because in his opinion restricting histology to organs with gross lesions significantly decreases the determination of complete toxicity. He also rejects the procedures of examining only the liver and kidney since morphological effects or target organs for new drugs are unknown; of examining only the major organs because this is too imprecise and varies with the pathologist's definition of major; and of examining organs with gross lesions and all organs from a few representative test animals as this may not yield the optimum amount of morphological data. Benitz does allow for deviations from histopathological examination of all organs if previous studies for that chemical indicate certain toxicity patterns or if results are needed only for certain organ systems. He stressed, however, that all organ systems have demonstrated toxic effects from chemicals, as is shown in Table 4.33. These effects can be characterized as follows: 76.5% were degenerative changes, 14% were inflammatory lesions, 7% were circulatory disorders, and 1.8% were neoplastic, precancerous lesions. Benitz generally recommended weighing of the organs as

Table 4.33. Incidence (in percent) of drug-induced pathological changes encountered over a ten-year period in approximately 14 000 animals

System and organ	Incidence	
	By organ	By system
Cardiovascular system		4.3
Respiratory system		5.1
Alimentary system		20.6
Liver	15.8	
Gastrointestinal tract	3.8	
Pancreas	1.0	
Urinary system		13.0
Kidney	12.0	
Ureter and urinary bladder	1.0	
Reproductive system		7.7
Males	4.8	
Females	2.9	
Ductless glands		22.1
Thyroid	11.5	
Adrenal	4.8	
Pituitary	5.3	
Islets of Langerhans	0.5	
Hematopoietic system		3.3
Lymphoid and reticuloendothelial system		7.2
Central nervous system		3.3
Sensory system		1.9
Eye	1.4	
Ear	0.5	
Locomotor system		10.5
Skin		1.0

Source: Adapted from Benitz, 1970. Reprinted with permission of the publisher.

an indicator of toxicity. However, he felt that use of relative organ weights could be misleading, and if they are used the absolute organ and body weights should also be given. This eliminates any uncertainty regarding whether the change is caused by reduced body weight or actual organ weight change.

Benitz, in his discussion of the significance of organ weight changes, deals with the question raised by Peck (1968) concerning how often organ weight changes correspond with positive histopathology findings. Benitz cited Jackson and Cappiello (1964) and their conclusion that in beagles 80% of the organ weight changes correlates with histopathological damage. In assessing the extent of histological damage, Benitz discussed both the role of electron microscopy and the quantification of morphological changes. He felt electron microscopy is useful for: (1) determining the earlier stages of morphological changes, (2) better interpreting visible lesion damage, and (3) detecting ultrastructural changes not visible through normal techniques. However, because of the time and effort involved, the use of electron microscopy must be adequately justified in each instance. Quantification of morphological changes is of value because results are more precise and comparable. Table 4.34 describes several generally used quantification methods. As can be discerned from his lengthy treatment of histopathology, Benitz felt it is highly important to the determination of toxicity. Although some of this may be due to his consideration of chronic as well as subchronic tests, the principles discussed apply equally to both topics.

Changes in body weight can often give misleading results for relative organ weight change. This can lead to errors in assessing toxic effects. Stevens (1976, 1977) examined this problem and proposed a simple modification of the standard relative weight determination. By obtaining initial and terminal body and organ weights for untreated test animals, Stevens constructed a simple linear regression of body weight and organ weight. This regression is used to predict the expected mean organ weight for a test group based on their mean body weight. Comparisons can be made between the expected and the actual relative body weights. Differences

Table 4.34. Summary of histological quantification methods and their applications for chemically induced lesions

Procedure	Histological substrate	Comments
Counts	Bile plugs, giant cells, mast cells, calcification sites, mitoses, etc.	Expressed as average number per field; total number per section, etc.
Linear measurements	Thinning of femoral epiphyseal disc; hyperplasia of zona glomerulosa in adrenal cortex; reduction in height of spermatogenic epithelium; decrease of tubular diameter of testes	Calibrated micrometer, results in micrometer units or microns
Area measurements	Muscles: necrosis Uterus: atrophy of mucosa and myometrium Brain: hydrocephalus	Components expressed in planimeter values obtained from tracings or projections
Nuclear volume	Hyperplasia of thyroid gland; dystrophy of adrenal cortex; atrophy of liver due to starvation	Two perpendicular measurements using computerized formula of rotation ellipsoid for calculating μ^3
Composition of organs	Hyperplasia of thyroid gland: epithelium colloid, open capillaries, etc. Atrophy of ovaries: ratio of primary follicles to corpora lutea Cirrhosis of liver: density of reticulum fibers Composition of submaxillary glands: ratio of mucous to serous terminal portions	Point sampling,[a] Components expressed in percentage of organ volumes
Nuclear density	Dystrophy in zona fasciculata of adrenal cortex	
Internal surface measurements	Alveolar surface in lungs altered by inflammation, fibrosis, or emphysema. Trabecular surface in femoral epiphysis altered by osteodystrophy	Line sampling[a] Results expressed in mm^2/mm^3 of organ volume

[a] For methodology and examples see Hennig, 1959, and Benitz and Dambach, 1964.

Source: Adapted from Benitz. 1970. Reprinted with permission of the publisher.

indicate the actual toxic effect. By analyzing simulated results from rat organ weights, Stevens was able to test this modification and found it to be a significant improvement in relative organ weight data.

Several recent committee reviews of toxicity tests have discussed procedures for pathology examinations. The National Academy of Sciences review (1977) suggested that as a minimum all organs of animals at the high dose and controls should be examined microscopically. Further tissue examination at other dose levels should be based on gross observations and results from the minimal microscopic examinations. The Food Safety Council (1978) also recommends this procedure, with additional emphasis on gross examination.

The review by the World Health Organization (1978) is more extensive in its discussions. It generally recommends the procedures endorsed by the National Academy of Sciences and Food Safety Council, but points out more hazards in data evaluation. In interpreting the data, the pathologist should be aware of the rate of occurrence for spontaneous lesions and allow for these in his examination. Also organ weight changes should be evaluated carefully to determine if they resulted from the toxin or as a consequence of stress phenomena or metabolic overloading. Finally, they emphasize that microscopic examinations should be performed only after all data from the gross examination, organ weight determinations, and biochemical-hematological tests have been properly interpreted. These data will help direct the microscopic examination and reduce redundant efforts.

The U.S. Environmental Protection Agency (1979), in a support document for chronic toxicity testing guidelines, follows the recommendations of the National Cancer Institute (Sontag, Page, and Saffiotti, 1976), which suggested microscopic examination of all test animals (both control and treatment groups) in chronic studies. For subchronic studies, Sontag, Page, and Saffiotti (1976) suggested that all animals from the control, highest-dose, and next highest-dose levels be subjected to a histopathologic examination covering 30 to 40 tissues plus tissues with gross lesions. They feel the results obtained with this added pathology are worth the increased time and costs, which can account for 40% of the overall study cost (Page, 1977). This represents the maximum recommended microscopic examination recorded in the literature.

4.5.3 Basis for a Minimum Pathology Screen

The value of histopathology in subchronic tests is related to the sensitivity of each organ to different chemicals. If certain organs consistently show toxic effects in subchronic tests, they should be routinely included in most histopathologic examinations. These organs could comprise a minimum, basic pathology screen to which other organs could be added depending on the suspected toxic effects from the test chemical.

To aid in evaluating this concept, a rough tabulation of organ testing frequency and frequency of positive findings was constructed from 54 subchronic studies (Table 4.35). These subchronic studies tested a wide range of chemicals (Appendix B), including various drugs, herbicides, pesticides, fungicides, industrial chemicals and solvents, food additives, resins, and natural substances. The cumulative data for each organ are given in this table, combining three species (dog, rat, and monkey) and three routes (oral, inhalation, and injection). Appendixes B and C contain specific information on routes of exposure, test species, chemicals used, and literature references for each organ in Table 4.35. The table gives an indication of how often each organ is examined and how informative it is in subchronic studies. The "total studies" column refers to the number of studies in the literature that were examined for these data. The "studies using organ" column refers to the actual number of studies for which that specific organ was examined for pathologic changes. The "positive results" column refers to the number of studies in which pathologic changes were found in that organ. The "percent used" column refers to the number of studies using that organ as a toxicity indicator, out of the total number of studies reviewed. The "percent positive" column refers to the percentage of studies finding pathological effects in an organ out of the total number of studies using change in that organ as a criteria of toxicity. For example, the brain was evaluated for pathologic changes in 46 of 54 studies for a

Table 4.35. Comparison of the usefulness of individual organs to indicate toxicity when pathologically examined in a subchronic study

Organ[a]	Total studies[b]	Studies using organ[c]	Positive results[d]	Percent used[e]	Percent positive[f]
Adrenal	54	46	13	85	28
Aorta	54	17	0	31	0
Bone	54	13	1	24	8
Bone marrow	54	22	2	41	9
Brain	54	46	7	85	15
Esophagus	54	18	0	33	0
Eye	54	14	0	26	0
Gallbladder	54	9	1	17	11
Heart	54	53	8	98	15
Kidney	54	53	29	98	55
Large intestine	54	38	3	70	8
Liver	54	54	28	100	52
Lung	54	50	15	93	30
Lymph node	54	31	5	57	16
Mammary gland	54	8	0	15	0
Muscle tissue	54	22	0	41	0
Nerve tissue	54	15	6	28	40
Ovaries	54	36	6	67	17
Pancreas	54	36	1	67	3
Pituitary	54	26	1	48	4
Prostrate	54	19	3	35	16
Salivary gland	54	26	2	48	8
Sciatic nerve	54	4	1	7	25
Skin	54	11	1	20	9
Small intestine	54	39	1	72	3
Spinal cord	54	16	4	30	25
Spleen	54	49	12	91	24
Stomach	54	38	3	70	8
Testes	54	40	12	74	30
Thymus	54	24	6	44	25
Thyroid	54	36	5	67	14
Trachea	54	18	4	33	22
Urinary bladder	54	33	0	61	0
Uterus	54	20	1	37	5

[a] Appendices B and C contain specific information for each organ concerning test species, routes of exposure, chemicals tested, and literature references.

[b] Total number of studies reviewed for the inclusion of an organ as an indicator of pathologic changes.

[c] Total number of studies in which an organ was used to assess pathologic changes.

[d] Number of studies in which pathologic changes were detected in an organ.

[e] Frequency that an organ was used as a pathologic indicator (b/a × 100).

[f] Frequency that positive pathologic changes were found in an organ (c/b × 100).

frequency of use percentage of 85. However, out of the 46 studies utilizing the brain in this manner only 7 showed positive pathological data. Thus, the frequency of positive results for the brain was 15%. With the relative criteria of a 20% use frequency and a 5% positive result frequency, a minimum screen would consist of the following organs: adrenal, bone, bone marrow, brain, heart, kidney, large intestine, liver, lung, lymph node, nerve tissue, ovaries or testes, prostate, salivary gland, skin, spinal cord, spleen, stomach, thymus, thyroid, trachea, and uterus. This minimum screen does include representative organs from all of the major systems, except for sensory (e.g., eye) and would seem to be a logical start for composing a pathology evaluation.

4.5.4 Conclusions

In reviewing the literature on the use of pathology, the wide variety of protocols is striking. In few cases do the discussions agree concerning the amount and type of pathology necessary. The opinions regarding the value of gross examination are perhaps the closest to a consensus. Most reviews recommend gross examination of all organs at all dose levels (Barnes and Denz, 1954; National Academy of Sciences, 1977; Peck, 1968; World Health Organization, 1978), although the definition of *all organs* is often not specified. This procedure would seem to be the best option, as time and cost factors are not excessive and the information gained can be substantial (Food Safety Council, 1978).

The recommendations for microscopic examination are not as simple or similar as for the gross examination. Initially the importance of microscopic examination for subchronic tests was rated low, with few tissues examined (Smyth and Carpenter, 1948; Smyth, Carpenter, and Weil, 1951; Weil and McCollister, 1963; Weil et al., 1969). As toxicity tests became more extensive, the role of microscopic observations became greater. Many reviewers recommend microscopic examination of selected tissues (25 or less) at the high- and control-dose levels (Barnes and Denz, 1954; Zbinden, 1963). These tissues are usually major organs and lesion-bearing organs observed in gross examination. Other researchers believe that all organs (30 to 40) should be sampled at high and control doses to adequately determine toxicity (Benitz, 1970; Food Safety Council, 1978; National Academy of Sciences, 1977; World Health Organization, 1978). Yet other researchers believe that this is still insufficient and recommend examination of all tissues at all levels (Sontag, Page, and Saffiotti, 1976; U.S. Environmental Protection Agency, 1979). This last protocol is obviously the most sensitive in detecting toxicity, but it is also the most costly. The data in Table 4.35 and the recommendations of other researchers also indicate that the examination of all tissues at two dose levels can be excessive, because some tissues are poor indicators of toxicity.

The majority of researchers agree that a highly efficient protocol, in terms of toxicity detection per time and personnel cost, is the microscopic examination of specified organs and tissues at the high- and control-dose levels with added examination of grossly visible lesion-bearing organs. The exact number and type of tissues to be examined would vary with the chemical and its suspected target organs, but, at the least, every organ system should be represented.

The value of organ weight determinations is another controversial aspect of pathology. Although on occasion changes in organ weights may not indicate specific toxicity, since they may reflect problems such as functional hypertrophy or metabolic overloading, they may still provide information valuable in a toxicological assessment. Furthermore, many researchers feel that it can be more signifcant than histopathology. Since the procedures for determining organ weight (relative or absolute) are relatively easy, the most efficient protocol would include them. Even if considered insufficient alone as toxicity measures, changes in organ weight can indicate the general trends and suggest specific organs for microscopic examination.

Thus, the data presented in the literature surveyed indicate that an efficient, yet relatively inexpensive, pathology protocol would consist of a gross examination of all tissues and organs at all dose levels, a microscopic examination of designated tissues at the high and control levels, organ weights for major organs, and supplemental microscopic examination of tissues at other dose levels based on the gross observations and the organ weight data.

4.6 CLINICAL LABORATORY TESTS

4.6.1 Introduction

Recent developments in automation, methodology refinement, and data interpretation have greatly increased the predictive role of biochemical tests on blood, urine, and enzyme systems. Almost all current testing schemes include some biochemical tests requiring at least pre- and post-treatment sample evaluation. If more thorough toxic effect data are required, interim sacrifices or periodic sample removals are needed to provide sufficient data for evaluation. Despite automation, this increases time and cost factors in subchronic toxicity tests. The EPA guidelines for pesticide evaluations (*Federal Register*, 1978) recommend the following 31 biochemical tests: hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, platelet count, reticulocyte count, serum calcium, serum potassium, serum lactate dehydrogenase (LDH), SGPT, SGOT, glucose, blood urea nitrogen, direct and total bilirubin, serum alkaline phosphatase, total cholesterol, albumin, globulin, total protein, serum chlorine, uric acid, blood creatinine, urine specific gravity, urine pH, urine protein, urine glucose, urine ketones, urine bilirubin, and urobilinogen. In addition to these, some toxicologists also use the following determinations to assess toxic effects: ornithine carbamyl transferase, creatinine phosphokinase, lipase, amylase, sodium, prothrombin time, serum thyroxine, cholinesterase activities, packed cell volume, mean corpuscle hemoglobin, mean corpuscle volume, glucose-6-phosphatase, hexobarbital oxidase, methemoglobin, and many others. All of these tests are used to enhance early detection of toxicity or to help pinpoint toxic mechanisms. However, the actual value of this large number of tests has been discussed infrequently in the literature. The purpose of this section is to summarize the available literature on the use of hematological, biochemical, enzymatic, and urinalysis tests in subchronic tests and to present some data on the predictive efficiencies of these tests.

4.6.2 General Clinical Testing

Weil and McCollister (1963), in their comparison of short- and long-term feeding studies, evaluated the efficiency of clinical urinalysis, blood urea nitrogen, and hematology as primary indicators of toxic effects. As shown in Table 4.32, of 24 pertinent studies using these criteria only one showed any effects. Furthermore, the effect noticed was not at the lowest toxic dose, indicating that it contributed only secondary toxicity information. They concluded that efficient detection of toxicity did not need these types of tests for experiments of a subchronic nature.

Peck (1968), in reviewing the state of drug safety evaluations, commented on the value of biochemical, hematologic, and function tests. He cited the Coggeshall *Report of the Committee on Drug Safety* (1964), attributing the origin of biochemical toxicity tests as beginning with disease evaluation in man. These tests, originally designed for human systems, were incorporated in evaluations of animal toxicity effects, with an assumption that the tests would give comparable results in both man and test species. Both the Coggeshall report and Peck questioned the original validity of this assumption, especially without verifying studies. Peck also stated that the interpretation of data from these tests needs improvement and refining.

The use of tests dealing with changes in organ function or enzymatic activities for drug safety evaluations without supporting pathological data is especially questionable. Here Peck's opinion is essentially the same as his opinion on using organ weight changes without correlating histopathology. In both situations, it is necessary to utilize data from several methods of toxicity assessment if the evaluation is to be conclusive. Peck felt that experience has shown that the tests are generally valid for man and the test species, but any toxicity study should consider the test development history in making evaluations or recommendations.

The occurrence of abnormal results is also a problem in the interpretation of biochemical or function tests. Average values within two standard deviations and a knowledge of past aberrant biochemical test values for the species under study are essential to a proper evaluation of data. This will help eliminate concern over spontaneous abnormal values. If these factors are considered and compensated for, then the use of biochemical, hematologic, and function tests can be valuable in determining toxicity.

Benitz (1970), in a review of chronic toxicity, discussed the value of clinical chemistry, hematology, and serum drug levels in evaluating toxicity. In contrast to some authors, he stated that a large battery of tests is not always better than a smaller set. If the small set is constructed of tests better suited to evaluate the chemical's toxicity, as indicated in short-term tests, the results will be more meaningful. Use of interim function and clinical tests is helpful primarily in aiding the postmortem evaluation, because these tests indicate the sites of toxic damages. Benitz also suggested the use of tests similar to those used in humans, with the stipulation that they be equally effective. This agrees in principle with Peck's (1968) experiences in the use of human tests in experimental species. As an aid in evaluating the test results, Benitz suggested that baseline studies be done prior to testing rather than using "normal" values from the literature or other laboratories. Another important role of the pretest baseline examination is to determine the health status of the test animal, which allows the researcher to remove any potentially questionable subjects before treatment starts.

In assessing hematological data, one should be aware of the variability between samples taken from different sites in the vascular system and of differences in reliability between methods. In addition to numerical data, morphological or qualitative data can aid in assessing toxicity. Serum levels of drugs are most useful for determining toxic dose level and for comparison with levels to be used in man. By assessing the serum level at which toxic effects occur, comparisons between human clinical trials and animal toxicity studies are facilitated. In addition, Benitz warned against a rigid predetermined schedule of sampling. He stated that "these observations should be made when necessary." "When necessary" is determined during the trial by toxic effects from the high dose and from pretest short-term studies. To set up an inflexible schedule of sampling only invites unnecessary evaluations and a waste of time and money. In conclusion, Benitz supported Peck's (1968) association of pathology evaluations and clinical-hematologic tests, stating that toxic effects are not always indicated only by morphological damage or only by functional impairment.

McNamara (1976) briefly discussed hematology and biochemical tests, indicating that he supported their inclusion in a test design. He listed these tests as minimal requirements: blood urea nitrogen; sugar, glutamic oxalacetic transaminase; alkaline phosphatase; glucose-6-phosphatase; cholinesterase; lactic acid dehydrogenase; potassium (albumin, globulin); sodium; calcium; chloride; carbon dioxide; hematocrit; hemoglobin; total and differential white blood cells; and erythrocytes. He also suggested that these tests be performed at exposure days -14, -7, +3, +7, +30, +60, and +90. This schedule allows for establishment of pretreatment baseline values and ensures a sufficient number of determinations to detect most transient changes.

The National Academy of Sciences (1977) review on toxicity testing briefly discussed biochemical and hematological examinations. These investigators suggested selected use of clinical tests based on prior test data or chemical class. They agreed with Benitz (1970) in recommending that a rigid schedule of sampling is unnecessary; a flexible schedule that can be modified by the health status of the test animals is more practical. They suggested using large animals (nonrodent) for most tests, especially organ function tests or special clinical examinations (e.g., x rays or respiratory rate determinations). They did not recommend any specific tests, suggesting that this be left to the investigators.

The Food Safety Council (1978) stated that biochemical and hematological examinations should be a part of every study, with the degree of emphasis dependent on the substance. They also recommended a flexible sampling schedule, with shorter intervals between samples when the toxicity potential is unknown or unpredictable. They did not recommend any specific set of tests but suggested a variety of tests including some biochemical, hematological, urine, and fecal evaluations. However, in contrast to other reviews, they did not recommend baseline studies but suggested that historic colony values are acceptable. They also suggested that the final protocol be determined by the investigator.

In addition to these general comments on clinical tests, there are comments in the literature concerning specific hematology, organ/enzyme function, and urinalysis evaluations, which are discussed in the following sections.

4.6.3 Hematology

Zbinden (1963) discussed in his review of drug toxicity the various tests used for evaluations of hematological toxicity. For evaluation of total hematological effect, he recommended starting with a group of routine tests (hemoglobin, hematocrit, total red blood cells, total leucocytes, and differential leucocytes). As the study progresses, special tests (reticulocyte counts, Heinz-Ehrlich bodies, methemoglobin, sulfhemoglobin, thrombocyte count, and sedimentation rate) are added when indicated. The tests should be performed at monthly intervals, evaluating some animals from all dose levels for nonrodents and from the high-dose level for rodents.

Saslaw and Carlisle (1969) reviewed their toxicity data on various chemicals and drugs in which they utilized nonhuman primates (primarily rhesus monkeys) and monitored hematology parameters. They determined that for hematology effects the monkey was a good animal model for predicting effects in humans. Also, they discussed the value of hematology tests in determining general toxicity. In their opinion, hematology should be included in toxicity tests, as the laboratory work is not too excessive and "this effort could possibly be rewarded with observations that could be meaningful to groups or agencies faced with the awesome task of predicting or assessing hematoxicity of the increasingly complex preparations intended for eventual use in man." However, few definitive studies in support of this statement are included in the discussion. Saslaw and Carlisle also commented on the need for preliminary baseline studies for each specimen to be used in the toxicity tests. This would supply significant pretreatment "normal" values for the blood factors, which would allow easier assessment of changes occurring during treatment. The use of "mean average count" data is not as efficient as the individual baseline data, and its use should be discouraged.

Bushby (1970) evaluated the role of hematological tests in toxicity studies. In subchronic studies, the essential hematological tests should detect anemia and alterations in leucocytes and platelets. If these changes are observed, more testing should be performed to determine the causes. Bushby broke these two types of hematology tests (detection of effects tests and site of action tests) into the three groups listed in Table 4.36.

To detect anemia, Bushby recommended a hemoglobin count, a red blood cell count, and a determination of the packed cell volume. All three tests are interrelated, and a change in any of the three would indicate a possible anemia. Determination of the packed cell volume is easier and more reliable than the red blood cell count, so it is preferred. Additional tests that can pinpoint the type of anemia or site of action include mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, circulating reticulocyte numbers, bilirubin, and methemoglobin. For detecting changes caused by organic disease, Bushby recommended use of the sedimentation rate of the red blood cell count, an easily performed procedure.

For detection of alterations in the leucocytes and platelets, Bushby suggested a direct count of their numbers. An examination of a stained blood film will supply information on any gross morphological changes in platelets and leucocytes. The type and number of leucocytes present can also be determined with the film. This differential leucocyte count should always be converted to absolute figures using the total leucocyte numbers. Bushby did not recommend a routine test for the determination of hemorrhagic disorders. If changes are indicated by clinical evidence or platelet counts, the prothrombin and partial thromboplastin times should be determined. Bushby concluded that in most prolonged studies a few hematological parameter evaluations will detect general blood damage but, if site of action information is desired, more extensive and different tests are necessary.

Table 4.36. Hematological studies during toxicity tests

Group

Primary tests and estimations

- Hemoglobin estimation
- Packed cell volume
- Total and differential leucocyte count
- Platelet count
- Examination of stained film for polychromasia and abnormal leucocytes and platelets
- Sedimentation rate

Secondary tests

- Red cell count
- Calculation of mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration
- Reticulocyte count
- Detection of Heinz bodies
- Examination for methemoglobin
- Hemoglobinemia
- Bilirubinemia
- Bone marrow
- Coagulation time
- Bleeding time
- Clot retraction
- Prothrombin time
- Partial thromboplastin (cephalin) test
- Fragility test

Follow-up investigations

- Schumm's test for methemoglobin
- Coproporphyrins I and III in urine
- Thromboplastin generation test
- Platelet adhesion
- Antiglobulin test for autoantibodies
- Tourriquet or vacuum test
- Osmotic fragility
- FIGLU test
- Folic acid and B_{12} deficiencies
- Estimation of life span of red cell
- Iron metabolism
  - Ferrioxamine test
  - Serum iron estimation
  - Serum iron-binding capacity
  - Siderocytes in bone marrow
- Estimation of haptoglobin
- Blood volume

Source: Adapted from Bushby, 1970. Reprinted with permission of the publisher.

Arnold et al. (1977) commented on hematological procedures in their discussion of animal health monitoring in chronic studies. Generally, they recommended testing on a preset schedule or in response to changes in animal health. They also recommended using, but did not specify, a basic test set with incorporation of additional tests when any of these conditions are observed: obvious clinical anemia; preliminary conditions of anemia (e.g., vaginal bleeding or hematuria); enlargement of the spleen, liver, or lymph nodes; and signs of physical trauma.

The monitoring of hematological effects should begin with a gross observation of the blood sample to detect changes such as altered color or transparency. Next, standard erythrocyte and leucocyte evaluations should be performed. Arnold et al. stressed the need for interrelated and overlapping evaluations, especially the correlation of data from cell counts and blood smear examinations. Similarly, differential leucocyte counts must be translated into absolute numbers and given with the total leucocyte counts. To screen for hemorrhagic effects, they recommended the use of prothrombin time (one stage) and partial thromboplastin time (activated). If additional information is needed, the platelets should be counted and assessed for morphological changes, and bleeding time should be determined. If these procedures are followed, hematology can be a valuable tool in determining the type and degree of toxicity.

Another review that provides some detailed comments on hematogical data is a World Health Organization (1978) report on evaluations of chemical toxicity. These investigators recommended sampling at 30-day intervals with subgroups of rodent and nonrodent test species. They stated that to adequately assess hematological parameters both quantification and morphology of the blood cells are needed. They recommended a standard protocol that included the determination of erythrocyte numbers, reticulocyte numbers, total and differential leucocyte numbers, hematocrit, and hemoglobin. The erythrocytes must also be examined for morphological changes. For evaluating hemorrhagic effects, a screen should consist of platelet counts, clot retraction, prothrombin time (one stage), and partial thromboplastin time (activiated). A more detailed evaluation of hemorrhagic effects includes factor assays, thrombin time, fibrinogin determination, platelet aggregation, prothrombin consumption time, and euglobulin clot lysis time.

4.6.4 Biochemical and Organ Function

Wróblewski and LaDue (1955) evaluated SGOT as an index of liver cell injury. They primarily examined human patients with varying types of liver diseases, but also included some rat studies. Among the aims of their paper was to discover if the degree of SGOT activity was related to liver cell destruction; whether SGOT activity in chronic liver disorders indicated active liver cell destruction; what correlation existed between SGOT activity and various tests of liver dysfunction; and whether changes in SGOT reflected the presence of liver cancers. After evaluating their data, Wróblewski and LaDue reached the following conclusions: a rough relationship exists between the amount of a toxin in the animal and the degree of elevation of SGOT; the SGOT activity does not alter at the same rate or in the same direction as other liver function tests; SGOT appears to be an index of liver cell injury and not liver cell function changes; and an increase in SGOT activity indicates the presence of liver metastases. Based on this report, SGOT determinations appear to be a useful index of liver damage.

In addition to discussing hematology tests, Zbinden (1963) reviewed the use of clinical chemistry and organ function tests. The tests he listed as commonly utilized included SGOT; SGPT; serum lactate dehydrogenase; blood urea nitrogen; blood glucose; serum cholesterol; alkaline phosphatase; thymol turbidity; serum bilirubin; bromosulfalein excretion; nonprotein nitrogen; and phenolsulfonphthalein. However, he had reservations about their usefulness, stating, "Despite the widespread use of these tests, there is still not enough information about their significance in toxicology." Liver function tests are slightly more reliable than most of the tests and usually give results in the pathological range that correlate well with the degree of organ damage. However, not all degenerative changes that occur in the liver are detected. Thus, the significance of these tests should not be overrated either. Their use should obviously be as a confirming or indicator test. Zbinden also stressed the usefulness of drug blood level determinations. These allow more reliable extrapolation of "safe" dose levels from test species to man.

Hoe and O'Shea (1965) evaluated the predictive efficiency of biochemistry tests for detection of kidney disease in the dog. They used known kidney lesion-producing chemicals to assess the degree of correlation between biochemical tests and histopathology. The biochemical tests evaluated included blood urea; blood creatinine; inorganic phosphate; serum protein; calcium; serum alkaline phosphatase; sodium; potassium; chlorine; cholesterol; SGPT; and SGOT. Each was tested in dogs after exposure to the chemicals. The results of the tests for calcium, chlorine, and serum proteins did not correlate with kidney damage and were of no predictive value. The evaluations of alkaline phosphatase, SGOT, and SGPT were inconclusive regarding kidney damage due to excessive interference resulting from liver damage. However, the other tests did correlate with kidney damage. Blood urea increased in a parallel fashion with kidney damage. However, the authors warned that other researchers (Bloom, 1954) have found that urea values do not always correlate in cases of nephritis. Creatinine also indicated kidney damage but not as reliably as id urea changes. Inorganic phosphate showed a correlation, as did sodium and potassium. But Hoe and O'Shea cautioned that interpretation of these tests is complex, and careful evaluation is necessary. In conclusion, the authors suggested that by combining the biochemical tests and using urinalysis a forecast of 90% accuracy can be achieved for kidney damage. They suggested that general protocols cannot be developed and that every case or chemical should be assessed on an individual basis.

Peck et al. (1967) discussed several aspects of the interpretation of serum enzyme tests. They warn against assuming that a change in the test value automatically indicates a toxic effect. The change could be indicative only of reversible pharmacologic or physiologic actions of the test chemical. To properly assess the significance of the changes, one should consider the magnitude of the change, its incidence rate in the test groups, the presence or absence of a dose-response pattern, the results after continued repeated exposure, and the results of pharmacology and pathology evaluations. Another problem to be aware of is the potential deleterious loss of blood with repeated sampling. To prevent this, special studies or additional sample groups should be used, rather than jeopardizing the main study by oversampling.

Street (1970) reviewed biochemical tests in toxicology and stated that their primary functions are as early indicators of toxicity and for identification of target organs and evaluation of reversibility of changes. Most of the techniques employed are modified from human tests and can be done on an automated basis. For proper evaluation of the results, initial values and values from concurrent control groups are needed for comparison. The initial values should be taken twice during the acclimation period. Normal values published in the literature are of limited value because of high variability. Every toxicologist should establish the "normal" values at his own laboratory using his methods and species. These can then be used to aid in evaluation.

The usefulness and shortcomings of individual tests are discussed, and Street recommended analysis techniques for each one. For liver damage, he suggested the use of SGOT, SGPT, and alkaline phosphatase evaluations. Additional tests that can add information on other effects besides liver damage include bilirubin and protein electrophoresis. Street also recommended the use of isocitric dehydrogenase in place of the dye tests, unless the dye tests can be designed to give full elimination curves. For the assessment of kidney damage, he recommended the blood urea test correlated with urinalysis results. Other tests reviewed include electrolyte determinations, blood lipids, and cholinesterase. In conclusion, Street cautioned that biochemical test results usually suggest the use of additional confirmatory tests and in any case their findings should be checked against histopathology for correspondence of effects.

Cornish (1971) reviewed the literature associated with the use of serum enzyme changes as indicators of toxic effects. Cornish did not limit his discussion to tests that indicate liver and kidney damage, but also reviewed tests that may indicate heart or lung damage. In general, he refrained from recommending specific tests for each organ, preferring to emphasize the advantages and disadvantages found by researchers for each test. Several factors of test design are repeatedly stressed in his discussion. A principal factor is the need for frequent sampling or sampling during the periods when the changes in serum enzyme levels would be most probable. If sampling is done at inappropriate times, many of the changes may be missed, since the toxicant stimulates a short-term elevation of serum levels. Despite its often transient nature, this immediate response can be a sensitive indicator of functional and/or morphological damage. Although this might require a predosing determination of enzyme level patterns or a more frequent sampling schedule after dosing, the enzyme tests require only small samples of serum and can eliminate the need for the more costly interim sacrifices of test animals used to determine the onset of organ damage. Also, the toxicologist should consider other factors, such as stress and alterations in dietary intake, that can affect the serum enzyme levels regardless of any organ damage.

In addition to the usual enzyme tests, Cornish also discussed the potential value of serum isoenzyme determinations. Isoenzymes usually have a distinctive distribution pattern for each organ, and the pattern found in the blood after a chemical insult can indicate damage to individual organs. Because most of the other enzyme tests are less specific concerning the organ damaged, Cornish feels that the use of isoenzyme determinations has a great potential in toxicology. In addition to pinpointing damage to specific organs, isoenzyme patterns can often be more sensitive indicators of overall tissue damage than other enzyme tests. However, as with serum enzyme tests, the schedule of sampling for isoenzymes is important, because the pattern can change with time and alter the resulting diagnosis.

Grice et al. (1971) compared the sensitivity of changes in SGOT, lactate dehydrogenase (LDH), and LDH isozyme patterns with histopathology to determine their usefulness in detecting liver and kidney damage. After exposure of four toxic chemicals in rats, the test results showed that morphological damage occurs at lower doses and before any change in the serum enzyme levels. The SGOT test was somewhat more sensitive than LDH, but lacked the specificity of the isozyme pattern. Grice et al. concluded that the serum enzyme levels and isozyme patterns could contribute supplemental information, but they are not substitutes for pathology evaluations.

Korsrud et al. (1973) also used histopathology to assess the sensitivity of changes in several serum enzymes to indicate liver toxicity. They used acute doses of three toxic chemicals and monitored the changes in isocitrate dehydrogenase (ICD), fructose-1-P aldose (F-1-P ALD), sorbitol dehydrogenase (SDH), glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transminase (GOT), malic dehydrogenase (MDH), LDH, glutamic dehydrogenase (GDH), fructose-1,6-diphosphate aldolase (F-1,6-diP ALD), and ornithine carbamyl transferase (OCT) in rats. As shown in Table 4.37, histopathology was repeatedly the most sensitive index. Of the enzyme tests, SDH was the most sensitive. The next most reliable indicators were ICD, GPT, GOT, and F-1-P ALD. Korsrud et al. identified several factors that could account for the differences in sensitivity, such as different intracellular locations of the enzymes in the liver, high control values for LDH, different release rates into the blood for each enzyme, different turnover rates for the enzymes, and the enzyme activity in the cell prior to release. The responses were also dependent on the time of sampling. Korsrud et al. concluded that SDH was the best choice for assessing minimal liver damage when using serum enzyme levels.

Gray et al. (1972), in an evaluation of the toxicity of clindamycin in dogs, discussed the value of serum transaminase tests. In particular, they concentrated on the predictive value of changes in SGPT. They felt that SGPT changes were not so much an indication of actual liver damage as an indication of liver intolerance. They based this on the role of SGPT in the transport efficiency of the membrane. They concluded that no definite morphological effect could be related to SGPT changes; therefore, SGPT could not be used as a predictor of liver damage.

Arnold et al. (1977) briefly commented on the usefulness of serum enzyme determinations. They cautioned that the removal of blood and the

Table 4.37. Relative sensitivity of liver enzyme tests

Test[a]	Lowest dose at which significant change occurred (mg/kg)		
	TA[b]	DMN[c]	DEA[d]
ICD	25.4	13.7	800
F-1-P ALD	25.4	13.7	800
SDH	9.4	5.1	800
GPT	25.4	13.7	800
GOT	25.4	13.7	800
MDH	68.6	37.0	800
LDH	68.6	37.0	1600
GDH	NC[e]	NC	1600
OCT	68.6	37.0	1600
F-1, 6-diP ALD	NC	NC	1600
Histopathology	3.5	1.9	400

[a] ICD — isocitrate dehydrogenase; F-1-P ALD — fructose-1-P-aldolase; SDH — sorbitol dehydrogenase; GPT — glutamic-pyruvic transaminase; GOT — glutamic-oxaloacetic transaminase; MDH — malic dehydrogenase; LDH — lactic dehydrogenase; GDH — glutamic dehydrogenase; OCT — ornithine carbamyl transferase; F-1, 6-diP ALD — fructose-1,-diphosphate aldolase.

[b] TA — thioacetamide.

[c] DMN — dimethylnitrosamine.

[d] DEA — diethanolamine.

[e] No change.

Source: Adapted from Korsrud et al., 1973.

trauma associated with it can significantly affect the test animal's health. Therefore, serum enzyme tests should be performed only when necessary. Also, many of the tests fail to detect minor damage or may reflect only transient changes. As a result of these disadvantages, the use of biochemical tests is limited to a confirmatory role.

These same disadvantages and cautions are also voiced by the World Health Organization (1978). They suggested the use of large test species, such as the dog, to reduce sample removal effects. They recommended a set sampling schedule with serum withdrawal prior to the test, 3 and 10 days after the start of dosing, and continuing at 30-day intervals. They concluded that, in general, these tests cannot detect minor or initial changes in organ function but can act as a guide for further assessments.

Clampitt (1978) investigated the predictive value of various enzyme tests for liver damage in the rat. He compared these results with histopathological evaluations to determine their effectiveness. He found that no single test gave an unquestionable indication of liver damage. Many of the conventional liver damage tests were not the most sensitive indicators of damage. These include alkaline phosphatase, alanine transaminase, and aspartate transaminase. Clampitt did recommend that to assess lipid metabolism changes, plasma cholesterol and triglyceride levels should be monitored. Also, the time at which blood samples are taken should be carefully monitored if transient effects are desired. In conclusion, Clampitt suggested that minimal changes can be detected if the "appropriate plasma constituents" are measured and used with knowledge of the "intracellular location of the diagnostic enzymes."

4.6.5 Urinalysis

Balazs et al. (1963) discussed the value of urinalysis in assessing renal damage. In general the authors believed that although kidney function tests are not easily adapted to routine toxicity studies using the rat, they are potentially very useful Their data suggested that the proximal convoluted tubules were the most vulnerable part of the kidney. Their tests were therefore designed to evaluate damage in this area. They found that the renal tests that provide the most reliable information were the Addis count, urinary glutamic oxaloacetic transaminase test (UGOT), and urinary concentration test. Blood urea nitrogen was erratic and less reliable than the UGOT test because of extrarenal interference. Also, the standard concentration tests were inappropriate, and the authors suggested using a 6-h determination. This allowed minor effects to be detected. Additional evidence of renal damage can be obtained from observation of renal epithelial cells and casts in the urine.

Hoe and O'Shea (1965) evaluated biochemical analysis of renal damage in the dog, including the value of urinalysis techniques. They recommended the use of urine specific gravity, proteinuria, and the presence of casts in the urine to assess renal damage. As with their recommendations on enzyme tests, Hoe and O'Shea believed a combination of urinalysis and function tests was necessary for a complete assessment of renal damage. In this way, 90% of the cases could be diagnosed by biochemical tests alone.

Urinalysis and its diagnostic value were discussed by Street (1970). The use of the simple tablet or dipstick analyses techniques in any capacity greater than a preliminary screen is questioned by Street. He feels that the more complex tests add significant information, especially for renal and hepatic damage. The recommended tests include observations on the color and volume, pH (by meter, not paper), specific gravity, protein electrophoresis, examination of deposits, and 16-h concentration tests (in the dog). Simple screen tests can also be performed to check for glucose, ketones, free hemoglobin, bile, and reducing substances. Unlike Balazs et al. (1963), Street did not recommend the UGOT and Addis counts as standard tests. The specific gravity and concentration tests are sufficient to detect most renal damage. Urinary ascorbic acid can be used to test for liver enlargement, but it requires a series of comparative assays. Overall, Street recommended the inclusion of urinalysis tests for a complete assessment of toxicity.

Grice (1972) reviewed some aspects of urinalysis, particularly tests derived from human renal assessments. In general he feels the tests to be used on small laboratory animals have not reached a similar level of sophistication and are not as valuable as for humans. A major problem, besides the interpretation of test results, is obtaining a suitable sample. Collection is difficult, since the test animals "will not urinate on command" and contamination with feces or other materials is hard to prevent. He also discusses other techniques to evaluate renal damage, such as renal cell excretion rates (useful for assessing the degree of injury) and palpation of the kidney (often more sensitive than either urinalysis or urinary enzyme tests). However, he concludes that presently the tests are less sensitive and less reliable than histopathology.

Wright and Plummer (1974) evaluated the excretion rate of four urinary enzymes (lactate dehydrogenase, alkaline phosphatase, acid phosphatase, and glutamate dehydrogenase) as indicators of acute kidney damage by administering several known nephrotoxic agents. The enzymes were selected for their utility as "markers" for renal injury to specific regions in the cell, and the changes were compared to baseline excretion rates. They found that lactate dehydrogenase and alkaline phosphatase were excreted at significantly higher rates after renal insult, and these increases seemed to correlate with renal damage. The other two enzymes were not appreciably affected. They concluded that excretion rates of some enzymes could effectively detect acute renal damage.

Cottrell et al. (1976) conducted similar studies on the value of excretion rates of creatinine, alkaline phosphatase, lactic dehydrogenase, and leucine aminopeptidase after acute and repeated-dose exposure to nephrotoxicants. They used both histology and histochemistry to evaluate the renal damage. They found that for acute exposure, all the enzymes but creatinine were affected. Lactic dehydrogenase was the most sensitive indicator. However, the excretion pattern after repeated exposure to the nephrotoxicants did not continue to indicate the increasing renal damage shown by histology and returned to normal rates after three days. Therefore, they concluded that currently the value of urinary excretion rates is confined to acute and very short-term exposures. Their utility in subchronic tests would be quite limited.

Berndt (1976) reviewed the tests necessary for confirmation of the kidney as a target organ. In addition to recommending the hematology tests and blood urea nitrogen and plasma creatinine tests, Berndt recommended a urinalysis determination. Specifically he stressed the use of changes in urinary glucose levels as a good indicator of renal damage. Also, the excretion of p-aminohipparate (PAH) and tetraethylammonia (TEA) can be detected in the urine to indicate damage. These tests are particularly good for evaluating the toxic effect on the nephrons. Other recommended tests include the volume and concentration determinations. Protein determinations will indicate the effects on the glomerulus. Berndt concluded that, in comparison with urinalysis, histological and anatomical techniques are the "least useful criterion for evaluation of renal damage."

Arnold et al. (1977) also discussed the use of urinalysis in toxicity diagnosis. They suggested collection of urine overnight or at 24-h interims for determinations of volume and the excretion patterns of calcium, phosphate, creatinine, chloride, sodium, and potassium. Fresh samples were needed, however, for pH, osmolarity, and the presence of malignant cells. As a routine examination, the authors suggested the use of strip tests covering pH, protein, glucose, ketones, blood, and bilirubin. Depending on the results, more specific tests or more complex analysis could be incorporated, including microscopic evaluations of urine sediments. These urinalysis tests will elucidate renal problems such as anuria, hematuria, or hypertrophic changes. Thus, the authors recommended the inclusion of at least preliminary examination of the urine for all toxicity assessments.

A recent review of renal function tests evaluated the effectiveness of changes in glomerular filtration rate (GFR), renal plasma (RPF) and red blood flow (RBF), total urinary protein excretion, urinary concentration, urinary acidification, urinary enzyme excretion rates, urinary sodium excretion rate, and microscopic examination of sediments as indicators of nephrotoxic effects (Diézi and Biollaz, 1979). The literature they reviewed indicated that for GFR determinations urea and creatinine excretion rates were poor tests, and clearance rates for marker substances were preferred; RBF and RPF were extremely difficult to interpret, especially for rodents; total protein and urinary concentration can be useful screens but do not give site-specific information; urinary enzyme excretion has been shown useful but because of a lack of correlation with increases in renal damage, its utility is uncertain; and sodium excretion and urinary sedimentation are useful only as screening tools. They concluded the predictive value for human toxicity of these elaborate tests is uncertain and their routine use is not suggested. Also, the difficulty of sample collection and interpretation of normal variations in values makes the general use of these tests less practical. However, these tests can be used for more detailed information when prior studies indicate renal damage or when the structure of the chemical to be investigated suggests possible renal damage.

U.S. Environmental Protection Agency investigators, in a support document for chronic tests (1979), stressed the value of urinalysis.

They recommended a routine screening (semiquantitative) with a more detailed follow-up for positive results. They suggested the use of urinalysis to detect early renal damage, as most enzyme function tests do not adequately assess this. Although the EPA document evaluated chronic test applications, the principles should also apply to subchronic applications.

4.6.6 Conclusions

Biochemical tests have become a large part of the modern toxicity evaluation. In general, they provide early indications of the presence of toxic effects and suggest specific target organs or contribute confirmatory evidence on the toxic effect. The wide variety of tests available has presented the toxicologist with the problem of test selection. Most of the literature reviewed did not suggest a standard list of required biochemical tests, but rather suggested the inclusion of hematology, enzyme, and urinalysis evaluations. This is perhaps a good policy because the effectiveness and applicability of the tests change with the toxicologist (particularly his experience with these tests) and the chemical to be examined.

For hematology evaluations there was general agreement that a set of basic hematology tests should provide information on cellular damage and hemorrhagic effects. To properly evaluate these parameters, several lists of tests were suggested. Most schemes used a small routine set of tests with more complex evaluations added as indicated by the results. The routine tests included morphological and quantitative evaluations of erythrocytes and leucocytes plus a temporal evaluation of hemorrhagic effects. The scheme in Table 4.36 is representative of this.

The question of baseline studies or normal values for comparison was discussed in terms of hematology tests, although it also applies to enzyme function tests. Most reviews suggested that for nonrodent species, baseline data compiled before treatment were more valuable. A pretreatment examination also indicates the health status of the test animals, confirming their suitability for test use. The use of normal values was less acceptable because of variations between individual test animals and between laboratories. If normal values are to be used, they should be developed at the individual laboratory. The same applies to the use of historic colony values for rat studies. The use of baseline or normal values for comparison would be in addition to the use of concurrent properly designed control groups.

The sampling schedule was another area of discussion. Many reviews recommended sampling at 30-day intervals with initial samples at 1 day and 2 weeks. Others stress the value of flexible schedules adaptable to changes observed in the animals' health. The best solution would be an initial schedule of routine tests performed on a 30-day basis that is flexible enough to incorporate additional tests as the study progresses. The effect of sampling is greater for rodents than for nonrodents, because of size and blood volume considerations. A recent paper by Cardy and Warner (1979) discussed the effect of sequential bleeding on rats with the removal of 1 mL of blood every 2 weeks over a 23-week testing period. They found that the hematological values were not significantly altered but that body weight gain was significantly reduced on this sampling regime. Thus, if frequent sequential samples are taken, consideration must be given to their effect on all parameters.

Enzyme and organ function tests are usually also performed when hematology samples are taken with the emphasis on detecting liver and kidney damage. The literature recommends several procedures for assessing these kinds of damage. Liver tests usually recommended include SGOT, SGPT, SDH, and alkaline phosphatase, with the emphasis varying among species and chemicals. Kidney evaluations were primarily by blood urea and creatinine tests associated with urinalysis results. As indicated by several reviews, these results should be interpreted carefully and only used in connection with histopathological follow-up.

The value of urinalysis is not quite as accepted as hematology or enzyme tests. The National Academy of Sciences (1977) saw little value in routine urinalysis tests. The use of urinary enzyme excretion changes is particularly limited for subchronic studies. However, other reviews suggested the use of at least a dipstick check of the urine as a preliminary screen. Based on evidence in several articles, it seems that detailed assessments of kidney damage are best evaluated by using urinalysis tests and a few enzyme determinations. If used, the urinalysis examination should check physical (e.g., volume and specific gravity), enzymatic (e.g., lactic dehydrogenase), and chemical (e.g., glucose and phosphate) parameters. However, because of problems with sample collection and interpretation of test results, the routine use of urinalysis is questionable and should be left to the discretion of the investigator.

Thus, the value of hematology, enzyme function, and urinalysis techniques is apparently sufficient to recommend that they be included in standard subchronic toxicity protocols. The extent that each is utilized should be left up to the investigator. Perhaps the best utilization of the biochemical evaluations would be in conjunction with the terminal pathology examination. With refinement, the biochemical tests might suggest target organs and types of effects that could be checked by pathology. The effectiveness would thus increase for both procedures.

REFERENCES FOR SECTION 4

Abrams, W. B., G. Zbinden, and R. Bagdon. 1965. Investigative Methods in Clinical Toxicology. J. New Drugs 5:199-207.

Alumot, E., E. Nachtomi, E. Mandel, and P. Holstein. 1976. Tolerance and Acceptable Daily Intake of Chlorinated Fumigants in the Rat Diet. Food Cosmet. Toxicol. 14:105-110.

Ambrose, A. M., P. S. Larson, J. F. Borzelleca, and G. R. Hennigar, Jr 1972. Toxicologic Studies on 3',4'-Dichloropropionanilide. Toxicol. Appl. Pharmacol. 23:650-659.

Anderson, M. E., R. A. Jones, R. G. Mehl, T. A. Hill, L. Kurlansik, and L. J. Jenkins, Jr. 1977. The Inhalation Toxicity of Sulfolane (Tetrahydrothiophene-1,1,-Dioxide). Toxicol. Appl. Pharmacol. 40:463-472.

Ansbacher, S., W. C. Corwin, and B.G.H. Thomas. 1942. Toxicity of Menadione, Menadiol, and Esters. J. Pharmacol. Exp. Ther. 75:111-121.

Arnold, D. L., S. M. Charbonneau, Z. Z. Zawidzka, and H. C. Grice. 1977. Monitoring Animal Health During Chronic Toxicity Studies. J. Environ. Pathol. Toxicol. 1:227-239.

Atkinson, R. M., J. D. Caisey, J. P. Currie, T. R. Middleton, D.A.H. Pratt, H. M. Sharpe, and E. G. Tomich. 1966. Subacute Toxicity of Cephaloridine to Various Species. Toxicol. Appl. Pharmacol. 8:407-428.

Aviado, D. M. 1978. Overwhelming Similarities and Minimal Differences in Toxic Responses of Rats and Dogs (Part I: 1974-1978 and Part II: 1966-1974) (unpublished).

Balazs, T. 1976. Assessment of the Value of Systematic Toxicity Studies in Experimental Animals. In: Advances in Modern Toxicology, Vol. 1, Part I: New Concepts in Safety Evaluation. M. A. Mehlman, R. E. Shapiro, and H. Blumenthal, eds. John Wiley and Sons, New York, pp. 141-153.

Balazs, T., A. Hatch, Z. Zawidzka, H. C. Grice. 1963. Renal Tests in Toxicity Studies on Rats. Toxicol. Appl. Pharmacol. 5:661-674.

Barnes, J. M., and F. A. Denz. 1954. Experimental Methods Used in Determining Chronic Toxicity. Pharmcol. Rev. 6:191-242.

Bein, H. J. 1963. Rational and Irrational Numbers in Toxicology. Proc. Eur. Soc. Study Drug Toxic. 2:15-26.

Bencko, V., V. Dvorak, and K. Symon. 1973. Organ Retention of Parenterally Administered Arsenic (Labelled with ^{74}As) in Mice Preliminarily Exposed to the Element in Drinking Water: A Study in Arsenic Tolerance. J. Hyg. Epidemiol. Microbiol. Immunol. (Prague) 17:165-168.

Bencko, V., and K. Symon. 1969. Dynamics of Arsenic Cumulation in Hairless Mice After Peroral Administration. J. Hyg. Epidemiol. Microbiol. Immunol. (Prague) 13:248-253.

Bencko, V., and K. Symon. 1970. The Cumulation Dynamics in Some Tissue of Hairless Mice Inhaling Arsenic. Atmos. Environ. 4:157-161.

Benitz, K. F. 1970. Measurement of Chronic Toxicity. In: Methods in Toxicology, G. E. Paget, ed. F. A. Davis Company, Philadelphia. pp. 82-131.

Benitz, K. F., and Dambach, G. 1964. Quantification of Drug-Induced Morphologic Changes with Hennig's Point- and Line-Sampling Methods Using Zeiss Integrating Eyepieces I and II. *Arzneimittel-Forsch.* 14:929-935.

Benitz, K. F., G.K.S. Roberts, and A. Yusa. 1967. Morphologic Effects of Minocycline in Laboratory Animals. Toxicol. Appl. Pharmacol. 11:150-170.

Berndt, W. O. 1976. Renal Function Tests: What Do They Mean? A Review of Renal Anatomy, Biochemistry, and Physiology. Environ. Health Perspect. 15:55-71.

Bloom, F. 1954. Pathology of the Dog and Cat. American Veterinary Publications, Inc., Evanston, Illinois.

Boyd, E. M. 1961. Toxicological Studies. J. New Drugs 1:104-109.

Boyd, E. M. 1968. Predictive Drug Toxicity: Assessment of Drug Safety Before Human Use. Can. Med. Assoc. J. 98:278-293.

Bratton, A. C., Jr. 1945. A Short-Term Chronic Toxicity Test Employing Mice. J. Pharmacol. Exp. Ther. 85:111-118.

Bushby, S.R.M. 1970. Haematological Studies During Toxicity Tests. In: Methods in Toxicology, G. E. Paget, ed. F. A. Davis Company, Philadelphia. pp. 338-371.

Bushby, S.R.M., P. Lechat, and R. Santarato. 1966. Haematological Investigations in the Toxicity Testing of Drugs. Results of a Questionnaire Circulated to Members of the European Society for the Study of Drug Toxicity. Proc. Eur. Soc. Study Drug Toxic. 7:208-215.

Cameron, G. R. 1952. Pathology of the Cell. Oliver and Boyd, Ltd., Edinburgh. 120 pp.

Cardy, R. H., and J. W. Warner. 1979. Effect of Sequential Bleeding on Body Weight Gain in Rats. Lab. Anim. Sci. 29(2):179-181.

Case, M. T., J. K. Smith, and R. A. Nelson. 1975. Reproductive, Acute and Subacute Toxicity Studies with Nefopam in Laboratory Animals. Toxicol. Appl. Pharmacol. 33:46-51.

Cavender, F. L., W. H. Steinhagen, C. E. Ulrich, W. M. Busey, B. Y. Cockrell, J. K. Haseman, M. D. Hogan, and R. T. Drew. 1977. Effects in Rats and Guinea Pigs of Short-Term Exposures to Sulfuric Acid Mist, Ozone, and Their Combination. J. Toxicol. Environ. Health 3:521-533.

Clampitt, R. B. 1978. An Investigation into the Value of Some Clinical Biochemical Tests in the Detection of Minimal Changes in Liver Morphology and Function in the Rat. Arch. Toxicol. Suppl. 1:1-13.

Coggeshall, L. T. 1964. Report of the Commission on Drug Safety. Library of Congress Catalog Number 64-16404, Washington, D.C. 228 pp.

Cornish, H. H. 1971. Problems Posed by Observations of Serum Enzyme Changes in Toxicology. Crit. Rev. Toxicol. 1(1):1-32.

Cottrell, R. C., C. E. Agrelo, S. D. Gangolli, and P. Grasso. 1976. Histochemical and Biochemical Studies of Chemically Induced Acute Kidney Damage in the Rat. Food Cosmet. Toxicol. 14:593-598.

Cummings, E. G., R. Armstrong, R. L. Farrand, and B. P. McNamara. 1979. Physiological and Behavioral Methodology for Evaluating Chemical Effects on Unanesthetized Rats. U.S. Army Armament Research and Development Command, Technical Report ARCSL-TR-79015. 33 pp.

Davey, D. G. 1964. The Study of the Toxicity of a Potential Drug: Basic Principles. Eur. Soc. Study Drug Toxic. Suppl. 6:1-13.

Diézi, J., and J. Biollaz. 1979. Renal Function Tests in Experimental Toxicity Studies. Pharmac. Ther. 5:135-145.

Earl, F. L., B. E. Melveger, J. E. Reinwall, G. W. Bierbower, and J. M. Curtis. 1971. Diazinon Toxicity — Comparative Studies in Dogs and Miniature Swine. Toxicol. Appl. Pharmacol. 18:285-295.

Earl, F. L., A. S. Tegeris, G. E. Whitmore, R. Morison, and O. G. Fitzhugh. 1964. The Use of Swine in Drug Toxicity Studies. Ann. N.Y. Acad. Sci. 111:671-688.

Fancher, O. E. 1978. Species Selection and Animal Models for Toxicologic Studies. Clin. Toxicol. 12(2):239-247.

Federal Register. 1978. Proposed Guidelines for Registering Pesticides in the U.S. Hazard Evaluation: Humans and Domestic Animals. U.S. Environmental Protection Agency [40 CFR Part 163] 43(163):37336-37403.

Feron, V. J., A. Kruysse, H. P. Til, and H. R. Immel. 1978. Repeated Exposure to Acrolein Vapour: Subacute Studies in Hamsters, Rats, and Rabbits. Toxicology 9:47-57.

Food and Agriculture Organization and World Health Organization Expert Committee on Food Additives. 1958. Procedures for the Testing of Intentional Food Additives to Establish Their Safety for Use. WHO Tech. Rep. Ser. No. 144. 19 pp.

Food Safety Council. 1978. Proposed System for Food Safety Assessment. Columbia, Maryland. 136 pp.

Freireich, E. J., E. A. Gehan, D. P. Rall, L. H. Schmidt, and H. E. Skipper. 1966. Quantitative Comparison of Toxicity of Anticancer Agents in Mouse, Rat, Hamster, Dog, Monkey, and Man. Cancer Chemother. Rep. 50(4):219-244.

Friedman, L., J. Sage, and E. M. Blendermann. 1970. Growth and Liver Response of Chicks and Rats to Carbon Tetrachloride and Ethanol. Poult. Sci. 49(1):298-309.

Gage, J. C. 1970. The Subacute Inhalation Toxicity of 109 Industrial Chemicals. Br. J. Ind. Med. 27:1-18.

Gaunt, I. F., G. Feuer, F. A. Fairweather, and D. Gilbert. 1965. Liver Response Tests. IV. Application to Short-Term Feeding Studies with Butylated Hydroxytoluene (BHT) and Butylated Hydroxyanisole (BHA). Food Cosmet. Toxicol. 3:433-443.

Gehring, P. J., V. K. Rowe, and S. B. McCollister. 1973. Toxicology: Cost/Time. Food Cosmet. Toxicol. 11:1097-1110.

Gray, J. E., R. N. Weaver, J. A. Bollert, and E. S. Feenstra. 1972. The Oral Toxicity of Clindamycin in Laboratory Animals. Toxicol. Appl. Pharmacol. 21:516-531.

Gray, T.J.B., K. R. Butterworth, I. F. Gaunt, P. Grasso, and S. D. Gangolli. 1977. Short-Term Toxicity Study of Di-(2-Ethylhexyl) Phthalate in Rats. Food Cosmet. Toxicol. 15:389-399.

Grice, H. C. 1972. The Changing Role of Pathology in Modern Safety Evaluation. Crit. Rev. Toxicol. 1(1): 119-152.

Grice, H. C., M. L. Barth, H. H. Cornish, G. V. Foster, and R. H. Gray. 1971. Correlation Between Serum Enzymes, Isoenzyme Patterns and Histologically Detectable Organ Damage. Food Cosmet. Toxicol. 9:847-855.

Guarino, A. M. 1979. Pharmacologic and Toxicologic Studies of Anticancer Drugs: Of Sharks, Mice, and Men (and Dogs and Monkeys). Methods Cancer Res. 17:93-174.

Hagan, E. C., W. H. Hansen, O. G. Fitzhugh, P. M. Jenner, W. I. Jones, J. M. Taylor, E. L. Long, A. A. Nelson, and J. B. Brouwer. 1967. Food Flavorings and Compounds of Related Structure. II. Subacute and Chronic Toxicity. Food Cosmet. Toxicol. 5:141-157.

Hall, D. E., P. Austin, and F. A. Fairweather. 1966. Acute (Mouse and Rat) and Short-Term (Rat) Toxicity Studies on Dibutyl (Diethylene Glycol Bisphthalate). Food Cosmet. Toxicol. 4:383-388.

Hals, E., G. Bjorlin, and K. Jacobsen. 1973. Histopathological Changes in Rat Incisors in Experimental Carbon Tetrachloride Intoxication. Odontol. Revy 24:5-26.

Harris, M. W., J. A. Moore, J. G. Vos, and B. N. Gupta. 1973. General Biological Effects of TCDD in Laboratory Animals. Environ. Health Perspect. 5:101-109.

Hartnagel, R. E., B. M. Phillips, P. J. Kraus, R. L. Kowalski, and E. H. Fonesca. 1975. A Subchronic Study of the Toxicity of an Orally Administered Benzoquinolizinyl Derivative in the Rat and Dog. Toxicology 4:215-222.

Hayes, W. J., Jr. 1967*a*. Toxicity of Pesticides to Man: Risks from Present Levels. Proc. R. Soc., B. 167:101-127.

Hayes, W. J., Jr. 1967*b*. The 90-Dose LD_{50} and a Chronicity Factor as Measures of Toxicity. Toxicol. Appl. Pharmacol. 11:327-335.

Hayes, W. J., Jr. 1972. Tests for Detecting and Measuring Long-Term Toxicity. Essays Toxicol. 3:65-77.

Hayes, W. J., Jr. 1975. Toxicology of Pesticides. Williams and Wilkins Co., Baltimore. 580 pp.

Hebold, G. 1972. Guidelines for the Testing of Drugs in Various Countries. In: World Congress of Anatomic and Clinical Pathology, M. Nordman, G. Menten, and H. Lommel, eds. Elsevier, New York. pp. 145-159.

Henning, A. 1959. A Critical Survey of Volume and Surface Measurements in Microscopy. *Zeiss-Werkz.* 6:3-12.

Hodge, H. C., A. M. Boyce, W. B. Deichmanne, and H. F. Kraybill. 1967. Toxicology and No-Effect Levels of Aldrin and Dieldrin. Toxicol. Appl. Pharmacol. 10:613-644.

Hodge, H. C., W. L. Downs, B. S. Panner, D. W. Smith, E. A. Maynard, J. W. Clayton, Jr., and R. C. Rhodes. 1967. Oral Toxicity and Metabolism of Diuron (*N*-(3,4-Dichlorophenyl)*N*',*N*'-dimethylurea) in Rats and Dogs. Food Cosmet. Toxicol. 5:513-531.

Hoe, C. M., and J. D. O'Shea. 1965. The Correlation of Biochemistry and Histopathology in Kidney Disease in the Dog. Vet. Rec. 77(8):210-217.

Homan, E. R. 1972. Quantitative Relationships Between Toxic Doses of Antitumor Chemotherapeutic Agents in Animals and Man. Cancer Chemother. Rep., Part 3. 3(1):13-19.

Hyslop, F., E. D. Palmes, W. C. Alford, A. R. Monaco, and L. T. Fairhall. 1943. The Toxicology of Beryllium. National Institutes of Health Bull. No. 181. 56 pp.

Jackson, B., and V. P. Cappiello. 1964. Ranges of Normal Organ Weights of Dogs. Toxicol. Appl. Pharmacol. 6:664-668.

Jones, R. A., J. A. Strickland, and J. Siegel. 1972. Toxicity of Propylene Glycol 1,2-Dinitrate in Experimental Animals. Toxicol. Appl. Pharmacol. 22:128-137.

Kast, A., Y. Tsunenari, M. Honma, J. Nishikawa, T. Shibata, and M. Torii. 1975*a*. Acute, Subacute, and Chronic Toxicity Studies of the Beta-Sympathomimetic, Fenoterol HBr on Rats, Mice, and Rabbits. Oyo Yakuri. Sendai 10(1):45-71.

Kast, A., Y. Tsunenari, M. Honma, J. Nishikawa, T. Shibata, and M. Torii. 1975*b*. Acute, Subacute, and Chronic Toxicity Studies of an Amino-Halogen-Substituted Benzylamine (Fominoben) in Rats and Mice. Oyo Yakuri. Sendai 10(1):31-43.

King, M. E., A. M. Shefner, and R. R. Bates. 1973. Carcinogenesis Bioassay of Chlorinated Dibenzodioxins and Related Chemicals. Environ. Health Persp. 5:163-170.

Knapp, W. K., Jr., W. M. Busey, and W. Kundzins. 1971. Subacute Oral Toxicity of Monochlorobenzene in Dogs and Rats. Toxicol. Appl. Pharmacol. Abstracts: Tenth Annual Meeting 19:393.

Kociba, R. J., P. A. Keeler, C. N. Park, and P. J. Gehring. 1976. 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD): Results of a 13-week Oral Toxicity Study in Rats. Toxicol. Appl. Pharmacol. 35:553-574.

Kociba, R. J., D. G. Keyes, J. E. Beyer, R. M. Carreon, C. E. Wade, D. A. Dittenber, R. P. Kalnins, L. E. Frauson, C. N. Park, S. D. Barnard, R. A. Hummel, and C. G. Humiston. 1978. Results of a Two-Year Chronic Toxicity and Oncogenicity Study of 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin in Rats. Toxicol. Appl. Pharmacol. 46-279-303.

Koeferl, M. T., C. D. Port, P. J. Garvin, and J. L. Dorner. 1976. Subacute Toxicity of Cyclohexanone in Rats, Dogs, and Monkeys. Toxicol. Appl. Pharmacol. Abstracts: Fifteenth Annual Meeting 37(1):115.

Kohn, F. E., J. H. Kay, and J. C. Calandra. 1965. Subacute Oral Toxicity of Zinophos. Toxicol. Appl. Pharmacol. Abstracts: Fourth Annual Meeting 7:488-489.

Korsrud, G. O., H. C. Grice, T. Kuiper Goodman, J. E. Kripfel, and J. M. McLaughlin. 1973. Sensitivity of Several Serum Enzymes for the Detection of Thioacetamide-, Dimethylnitrosamine-, and Diethanolamine-Induced Liver Damage in Rats. Toxicol. Appl. Pharmacol. 26:299-313.

Krasavage, W. J., F. J. Yanno, and C. J. Terhaar. 1973. Dimethyl Terephthalate (DMT): Acute Toxicity, Subacute Feeding and Inhalation Studies in Male Rats. Am. Ind. Hyg. Assoc. J. 34(1):455-462.

Kruysse, A., V. J. Feron, H. R. Immel, B. J. Spit, and G. J. Van Esch. 1977. Short-Term Inhalation Toxicity Studies with Peroxyacetyl Nitrate in Rats. Toxicology 8:231-249.

Lawrence, W. H., M. Malik, J. E. Turner, and J. Autian. 1972. Toxicity Profile of Epichlorohydrin. J. Pharm. Sci. 61(11):1712-1717.

Litchfield, J. T., Jr. 1961. Forecasting Drug Effects in Man from Studies in Laboratory Animals. J. Am. Med. Assoc. 177:104-108.

Litterst, C. L., T. E. Gram, E. G. Mimnaugh, P. Leber, D. Emmerling, and R. I. Freudenthal. 1976. A Comprehensive Study of *In Vitro* Drug Metabolism in Several Laboratory Species. Drug Metab. Dispos. 4(3):203-207.

Loomis, T. 1974. Essentials of Toxicology, 2nd edition. Lea and Febiger, Philadelphia. 223 pp.

Lyon, J. P., L. J. Jenkins, Jr., R. A. Jones, R. A. Coon, and J. Siegel. 1970. Repeated and Continuous Exposure of Laboratory Animals to Acrolein. Toxicol. Appl. Pharmacol. 17:726-732.

MacFarland, H. N. 1968. Exposure Chambers — Design and Operation. In: Proceedings of the 7th Annual Technical Meeting of the American Association of Contamination Control, Chicago, 1968. pp. 19-25.

McNamara, B. P. 1976. Concepts in Health Evaluation of Commercial and Industrial Chemicals. In: Advances in Modern Toxicology Vol. 1, Part i: New Concepts in Safety Evaluation, M. A. Mehlman, R. E. Shapiro, and H. Blumenthal, eds. John Wiley and Sons, New York. pp. 61-140.

McNerney, J. M., and J. D. MacEwen. 1965. Comparative Toxicity Studies at Reduced and Ambient Pressures. I. Acute Response. Am. Ind. Hyg. Assoc. J. 26:568-573.

Ministry of of Health and Welfare Canada. 1975. The Testing of Chemicals for Carcinogenicity, Mutagenicity, and Teratogenicity. Ottawa. 183 pp.

Misu, Y., T. Segawa, I. Kuruma, M. Kojima, and H. Takagi. 1966. Subacute Toxicity of *O*,*O*-Dimethyl-*O*-(3-Methyl-4-Nitrophenyl)phosphorothioate (Sumithion) in the Rat. Toxicol. Appl. Pharmacol. 9:17-26.

Morrow, P. E., H. C. Hodge, W. F. Newman, E. A. Maynard, H. J. Blanchet, Jr., D. W. Fasset, R. E. Birk, and S. Mavrodt. 1966. Deposition and Retention Models for Internal Dosimetry of the Human Respiratory Tract. Health Phys. 12:173-207.

National Academy of Sciences. 1975. Principles for Evaluating Chemicals in the Environment. National Academy of Sciences, Washington, D.C. 454 pp.

National Academy of Sciences. Committee for the Revision of NAS Publ. 1138. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. National Academy of Sciences, Washington, D.C. 130 pp.

Newberne, J. W., J. P. Gibson, and P. M. Newberne. 1967. Variation in Toxicologic Response of Species to an Analgesic. Toxicol. Appl. Pharmacol. 10:233-243.

Owens, A. H., Jr. 1962. Predicting Anticancer Drug Effects in Man from Laboratory Animal Studies. J. Chronic Dis. 15:223-228.

Page, N. P. 1977. Concepts of a Bioassay Program in Environmental Carcinogenesis. In: Advances in Modern Toxicology, Vol. 3: Environmental Cancer, H. F. Kraybill and M. A. Mehlman, eds. John Wiley and Sons, New York. pp. 87-171.

Page, N. P. 1979. Review of Current Activities in Test Standards Development. Presentation at EPA Subchronic Toxicity Workshop. Denver (unpublished).

Peck, H. M. 1968. An Appraisal of Drug Safety Evaluation in Animals and the Extrapolation of Results to Man. In: Importance of Fundamental Principles in Drug Evaluation, D. H. Tedeschi and R. E. Tedeschi, eds. Raven Press, New York. pp. 450-471.

Peck, H. M. 1974. Design of Experiments to Detect Carcinogenic Effects of Drugs. In: Carcinogenesis Testing of Chemicals, L. Goldberg, ed. CRC Press, Inc., Cleveland, Ohio. pp. 1-14.

Peck, H. M., P. A. Mattis, P. F. Stoner, and R. E. Zwickey. 1967. Toxicology. Drug. Inf. Bull. 1967:32-47.

Pinkel, D. 1956. The Use of Body Surface Area As a Criterion of Drug Dosage in Cancer Chemotherapy. Cancer Res. 18:853-856.

Prieur, D. J., D. M. Young, R. D. Davis, D. A. Cooney, E. R. Homan, R. L. Dixon, and A. M. Guarino. 1973. Procedures for Preclinical Toxicologic Evaluation of Cancer Chemotherapeutic Agents: Protocols of the Laboratory of Toxicology. Cancer Chemother. Rep., Part 3, 4(1):1-30.

Reddy, D. G., K. R. Krishnamurthy, and G. R. Bhaskar. 1962. Carbon Tetrachloride Cirrhosis in Rats. Arch. Pathol. 74:73-80.

Reeves, A. L. 1965. The Absorption of Beryllium from the Gastrointestinal Tract. Arch. Environ. Health 11:209-214.

Reeves, A. L., D. Deitch, and A. J. Vorwald. 1967. Beryllium Carcinogenesis. I. Inhalation Exposure of Rats to Beryllium Sulfate Aerosol. Cancer Res. 27 (Part 1):439-445.

Reuber, M. D., and E. L. Glover. 1967*a*. Cholangiofibrosis in the Liver of Buffalo Strain Rats Injected with Carbon Tetrachloride. Br. J. Exp. Pathol. 48:319-322.

Reuber, M. D., and E. L. Glover, 1967*b*. Thrombosis of Hepatic Veins Accompanying Carbon Tetrachloride Induced Cirrhosis. Arch. Pathol. 83:267-270.

Reuber, M. D., and E. L. Glover. 1967*c*. Hyperplastic and Early Neoplastic Lesions of the Liver in Buffalo Strain Rats of Various Ages Given Subcutaneous Carbon Tetrachloride. J. Nat. Cancer Inst. 38(1):891-899.

Reuber, M. D., and E. L. Glover. 1968. Carbon Tetrachloride Induced Cirrhosis. Effect of Age and Sex. Arch. Pathol. 85:275-279.

Robbins, G., and D. Tettenborn. 1976. Toxicity of Sisomicin in Animals. Infection 4 (Suppl. 4):349-354.

Roe, F.J.C. 1968. Inhalation Tests. In: Modern Trends in Toxicology, Vol. I., E. Boyland and R. Goulding, eds. Appleton-Century-Crofts, New York. pp. 39-74.

Rosenkrantz, H., R. A. Sprague, R. W. Fleischman, and M. C. Braude. 1975. Oral Δ^9-Tetrahydrocannabinol Toxicity in Rats Treated for Periods up to Six Months. Toxicol. Appl. Pharmacol. 32:399-417.

Rowe, V. K., H. C. Spencer, D. D. McCollister, R. L. Hollingsworth, and E. M. Adams. 1952. Toxicity of Ethylene Dibromide Determined on Experimental Animals. Arch. Ind. Hyg. Occup. Med. 6:158-173.

Saslaw, S., and H. N. Carlisle. 1969. Nonhuman Primates in Evaluation of Hematotoxicity. Ann. N.Y. Acad. Sci. 162(1):646-658.

Schein, P. S., R. D. Davis, S. Carter, J. Newman, D. R. Schein, and D. P. Rall. 1970. The Evaluation of Anticancer Drugs in Dogs and Monkeys for the Prediction of Qualitative Toxicities in Man. Clin. Pharmacol. Therap. 11(1):3-40.

Shimizu, Y., C. Nagase, and K. Kawai. 1973. Accumulation and Toxicity of Carbon Tetrachloride After Repeated Inhalation in Rats. Ind. Health 11:48-54.

Smith, C. C. 1950. A Short-Term Chronic Toxicity Test. J. Pharmcol. Exp. Ther. 100:408-420.

Smith, C. C. 1979. Extrapolation of Drug Metabolism Data from Animals to Man. In: Fundamentals and Principles of Clinical Pharmacology, H. P. Kuemmerle et al., eds. Urben and Swartzenberg, Baltimore (in preparation).

Smyth, H. F., Jr., and C. P. Carpenter. 1948. Further Experience with the Range Finding Test in the Industrial Toxicology Laboratory. J. Ind. Hyg. Toxicol. 30(1):63-68.

Smyth, H. F., Jr., C. P. Carpenter, and C. S. Weil. 1951. Range-Finding Toxicity Data: List IV. Arch. Ind. Hyg. Occup. Med. 4:119-122.

Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. U.S. Department of Health, Education, and Welfare Publ. No. (NIH) 76-801. 65 pp.

Stevens, M. T. 1976. The Value of Relative Organ Weights. Toxicology 5(3):311-318.

Stevens, M. T. 1977. An Alternative Method for the Evaluation of Organ Weight Experiments. Toxicology 7:275-281.

Stokinger, H. E., G. F. Sprague, R. H. Hall, N. J. Ashenburg, J. K. Scott, and L. T. Steadman. 1950. Acute Inhalation Toxicity of Beryllium. Arch. Ind. Hyg. Occup. Med. 1(4):379-397.

Stokinger, H. E., and C. A. Stroud. 1951. Anemia in Acute Experimental Beryllium Poisoning. J. Lab. Clin. Med. 38:173-182.

Street, A. E. 1970. Biochemical Tests in Toxicology. In: Methods in Toxicology, G. E. Paget, ed. F. A. Davis Company, Philadelphia. pp. 313-337.

Thompson, G. R., R. W. Fleischman, H. Rosenkrantz, and M. C. Braude. 1974. Oral and Intravenous Toxicity of Δ^9-Tetrahydrocannabinol in Rhesus Monkeys. Toxicol. Appl. Pharmacol. 27:648-665.

U.S. Environmental Protection Agency. 1979. Support Document Test Data Development Standards: Chronic Health Effects. Toxic Substances Control Act, Section 4, EPA 560/11-79-001, Washington, D.C. 154 pp.

Verschuuren, H. G., R. Kroes, and E.M.D. Tonkelaar. 1973. Toxicity Studies on Tetrasul II. Short-Term Comparative Studies in 6 Animal Species. Toxicology 1:103-112.

Verschuuren, H. G., R. Kroes, and E.M.D. Tonkelaar. 1975. Short-Term Oral and Dermal Toxicity of MCPA and MCPP. Toxicology 3(3):349-359.

Villeneuve, D. C., and W. H. Newsome. 1975. Toxicity and Tissue Levels in the Rat and Guinea Pig Following Acute Hexachlorobenzene Administration. Bull. Environ. Contam. Toxicol. 14(3):297-300.

Vogin, E. E., S. Carson, A. Palanker, and G. E. Cannon. 1970. Toxicologic, Reproductive, and Teratogenic Studies with a Tetracycline Phosphate Complex — Sulfamethizole Formulation. Toxicol. Appl. Pharmacol. 16:453-458.

Weil, C. S., and D. D. McCollister. 1963. Relationship Between Short- and Long-Term Feeding Studies in Designing an Effective Toxicity Test. J. Agric. Food Chem. 11(1):486-491.

Weil, C. S., M. D. Woodside, J. R. Bernard, and C. P. Carpenter. 1969. Relationship Between Single-Peroral, One-Week, and Ninety-Day Rat Feeding Studies. Toxicol. Appl. Pharmacol. 14:426-431.

Weisburger, J. H. 1975. Chemical Carcinogenesis. In: Toxicology: The Basic Science of Poisons, L. Casarett and J. Doull, eds. McMillan, New York. pp. 333-378.

Wiselogle, F. Y., ed. 1946. A Survey of Antimalarial Drugs: 1941-1945. J. W. Edwards, Ann Arbor, Michigan. 1863 pp.

Worden, A. N., and K. H. Harper. 1963. Oral Toxicity As Influenced by Method of Administration. Proc. Eur. Soc. Study Drug Toxic. 2:15-26.

World Health Organization. 1966. Principles for Pre-Clinical Testing of Drug Safety. WHO Tech. Rep. Ser. 341, Geneva. pp. 3-12.

World Health Organization. 1978. Principles and Methods for Evaluating the Toxicity of Chemicals: Part I. Environmental Health Criteria 6. Geneva. 273 pp.

Worth, H. M., D. B. Meyers, W. R. Gibson, and G. C. Todd. 1970. Acute and Subacute Toxicity of A204. Antimicrob. Agents Chemother. 357-360.

Wright, P. J., and D. T. Plummer. 1974. The Use of Urinary Enzyme Measurements to Detect Renal Damage Caused by Nephrotoxic Compounds. Biochem. Pharmacol. 23:65-73.

Wroblewski, F., and J. S. LaDue. 1955. Serum Glutamic Oxalacetic Transaminase Activity as an Index of Liver Cell Injury: A Preliminary Report. Ann. Intern. Med. 43:345-360.

Yeary, R. A., C. A. Brahm, and D. L. Miller. 1965. Acute and Subacute Toxicity of an Adduct of Hydralazine and a 3-Ketoalkylthiazide. Toxicol. Appl. Pharmacol. 7:598-605.

Zbinden, G. 1963. Experimental and Clinical Aspects of Drug Toxicity. Adv. Pharmcol. 2:1-112.

Appendix A

SUBCHRONIC TOXICITY TESTS AS DOSE-LEVEL-SETTING STUDIES FOR CHRONIC CARCINOGENICITY EVALUATIONS

David Prejean

INTRODUCTION

A subchronic toxicity test can be defined as an intermediate-length, repeated-dose study designed to indicate the potential long-term toxic effects of a chemical. If the study provides a sufficient basis for the estimation of safe exposure conditions, it may function as an "end result" study; however, if the data obtained are insufficient to estimate safety and a longer chronic study is required for evaluation, the subchronic toxicity test becomes a "dose-level-setting" study — the topic of this discussion.

Subchronic toxicity tests as dose-level-setting studies have a variety of applications, particularly as prechronic studies in teratogenicity, reproduction, longevity, or carcinogenesis evaluations; however, it is in the area of carcinogenicity that they have most recently been applied. Therefore, the discussion that follows centers on the use of subchronic toxicity tests to establish a maximum tolerated dose for each sex and species used in a chronic carcinogenicity study.

TEST MATERIAL

It is essential that the chemical being assayed be characterized as to purity and pertinent physical properties such as stability, solubility, and vapor pressure. To ensure consistency, it is preferable, when possible, to use the same manufacturer's lot number for both the subchronic and chronic studies. In addition, it is absolutely necessary to determine the accuracy and consistency with which the chemicals/vehicles are prepared (Page, 1977).

Although not all of the above requirements have been specified in performing the subchronic dose-level-setting studies in the past (National Institutes of Health, 1978), current specifications for conducting carcinogenesis bioassay studies, as set forth by both EPA (*Federal*

Register, 1978) and the National Cancer Institute (Sontag, Page, and Saffiotti, 1976), do require or recommend each of the above.

TEST ANIMALS

Species and Strain

Since the subchronic test is a dose-level-setting study, the species, strains, and sexes selected for use should be the same as will be used in the long-term study that follows. For chronic toxicity tests, two species have usually been recommended — preferably a rodent and a nonrodent (Page, 1977), with the rat and dog historically the most popular. Carcinogenicity studies, however, present a unique problem. Whereas toxicity studies, even long-term ones, require relatively small numbers of animals, carcinogenicity studies require a relatively large number for statistical purposes. Thus, because of the cost and time factors, small laboratory rodents, specifically the rat and mouse, have emerged as the most expedient test species.

As with the selection of the test animals for toxicity studies, the designation of a particular species to be used in a carcinogenesis study is only the beginning. Other questions, such as the use of inbred or random bred strains and the use of germ-free, specific pathogen-free, or conventional animals, must be answered. Although a large number of different rat and mouse strains have been utilized in the past (Boyland and Sydnor, 1962; Dunning, Curtis, and Madsen, 1947; National Institutes of Health, 1977; Pietra, Rappaport, and Shubik, 1961; Shimkin et al., 1962), the Fischer-344 rat and B6C3F1 hybrid mouse have become the standard for the National Cancer Institute Carcinogenesis Bioassay Program (National Institutes of Health, 1977; National Institutes of Health, 1978; Sontag, Page, and Saffiotti, 1976). Thus, although the controversy over the use of inbred or outbred strains continues, for the vast majority of long-term carcinogenicity studies performed between 1972 and 1979, the inbred and hybrid strains have been used.

The final consideration in the selection of a test animal has been the health status. For the most part, germ-free animals have rarely been used in chronic carcinogenesis testing because of the extensive labor requirements. Gnotobiotic, specific pathogen-free, and "extremely clean" conventional animals have been used most frequently in long-term carcinogenesis tests and thus have also been used most widely in subchronic tests designed to establish chronic dose levels (National Institutes of Health, 1978; Sontag, Page, and Saffiotti, 1976).

One final point should be made before leaving the area of species and strain. With rare exceptions, the majority of long-term carcinogenesis bioassay studies have been performed utilizing cost-effective systems rather than pursuing the question of carcinogenicity from the standpoint of the response of the test system to the chemical in question and the similarity to known responses in man. Future investigations should attempt to tailor the subchronic study to delineate one or more of a series of species and/or strains which would provide the most suitable test system.

Number

The number of animals required for a subchronic dose-level-setting study has varied considerably in the past. At one time the required number of animals per dose level was five animals per species, and sex was not taken into consideration (Prejean, 1979). Understandably, the resulting dose levels selected for chronic studies were, at best, guesses. In some cases the levels selected proved correct but, more often than not, incorrect dosages were selected, producing invalid chronic data.

The current trend in subchronic studies has been to use a minimum of ten animals of each sex and each species in the long-term study (Sontag, Page, and Saffiotti, 1976). This number has proved sufficient as long as the parameters for evaluation were limited to mortality, body weight gain, clinical signs, postmortem observation, and pathologic diagnosis; however, the recent expansion of these parameters to include hematology, clinical chemistry, and pharmacokinetic studies will, of necessity, produce an increase in the number of animals required for the subchronic study. There are indications that the role of the subchronic study in the carcinogenesis bioassay program is likely to require more animals, probably a minimum of 20 animals per sex per species (National Cancer Institute, 1979).

DOSES AND DOSE LEVELS

The doses selected for use in the subchronic study are usually based on data obtained from other shorter-term studies (Sontag, Page, and Saffiotti, 1976) but may, in some instances, be based on literature information. In selecting doses to be used in the subchronic study, it is important to remember that the ultimate objective of the study is to establish the maximum tolerated dose(s) for use in the chronic study. Thus, the more data available from the subchronic study, the more accurately the chronic dose levels may be set. For this reason, it is extremely important to select as the highest dose a level that will produce an easily discernible, reproducible toxic effect and a series of lower doses that provide a complete spectra of dose responses (Weisburger and Weisburger, 1967) from toxic to "nontoxic." In this way, not only can the dose levels for the chronic study be selected with more accuracy, but representative toxic lesions and symptoms may be observed that could be of value later in the chronic study.

Subchronic dose-level-setting studies should include, as a minimum, a range of five equally spaced dose levels (Sontag, Page, and Saffiotti, 1976). The intervals used most frequently have been half-doses (100, 50, 25, etc.) (Sontag, Page, and Saffiotti, 1976) or half-$\log_{10}$ doses (100, 31.6, 10, 3.16, etc.). Thus, assuming five dose levels, the lowest level tested would be approximately 6/100 of the top dose if half-doses are used or 1/100 of the top dose if half-$\log_{10}$ doses are used; either of these selections provides a reasonable span of doses from which to gather toxicity data.

ROUTE

Administration of the chemical in the subchronic study should closely simulate the route by which most human exposure occurs and should be identical to that which will be used for the chronic study.

With the exception of certain drugs, most chemicals enter the body by one of three routes — orally, by inhalation, or by absorption through the skin (Page, 1977). These then are the most common routes used to test chemicals for carcinogenicity. When exposure occurs by more than one route of administration, as in the case of pesticides, the route of exposure chosen is usually based on pharmacokinetic data, which could necessitate performing several subchronic studies before selecting the route and dose levels for the chronic study.

In general, chemical vapors and particulate materials such as smoke and asbestos are administered by inhalation, whereas food additives and pesticides are examples of chemicals that would be administered orally either by way of gastric intubation, the drinking water, or the feed. Cutaneous application, though not so common as oral administration, is the preferred route for administering cosmetics and certain drugs.

DURATION

Although the literature abounds with various specifications regarding the appropriate length for an end result subchronic study (Peck, 1968; Smith, 1950; Weil and McCollister, 1963), the majority of the evidence supports the conclusion that a 90-day test will predict most chronic effects exclusive of carcinogenicity and teratogenicity (McNamara, 1976). Since the function of a dose-level-setting subchronic test is to establish appropriate dose levels for a chronic study through the identification of subchronic toxic effects and their extrapolation to chronic toxic effects, a 90-day test should be just as appropriate for a dose-level-setting subchronic study as it is for an end result study. Of course, if prior information suggests that the chemical or its metabolites may be rapidly or slowly eliminated from the test species, then the duration may be reduced or extended as necessary. This has, in fact, proven to be the case. In the *Guidelines for Carcinogen Bioassay in Small Rodents* the National Cancer Institute recommends that in a subchronic study "The test agent should be administered to the animals for 90 days" (Sontag, Page, and Saffiotti, 1976). Thus, as with the other parameters already discussed, the duration of end result and dose-level-setting subchronic studies is compatible.

DATA COLLECTION

Of all the aspects of a subchronic test, regardless of whether it is an end result study or a dose-level-setting study, the most important is data collection. Without the accumulation of adequate and accurate observations during the test period and pathological examinations, the establishment of reliable chronic dose levels and/or the estimation of the long-term toxic effects is very difficult. What then are the important data collection points for a subchronic dose-level-setting study?

Clinical Observations, Body Weights, and Food and Water Consumption

During the course of a subchronic study much of the data must be collected on a daily or, at most, a weekly basis. Clinical observations as to the general health of each animal should be recorded twice daily, 7 days a week by a qualified technician (National Academy of Sciences, 1975). These observations should include an assessment of animal activity, posture, body temperature (extremes only), appearance of eyes and hair or coat, excreta and bedding, and respiratory function.

In addition to the above observations, each animal should be weighed weekly (Sontag, Page, and Saffiotti, 1976). Except in unusual instances, palpation for subcutaneous or intraperitoneal masses would not be required.

Food and water consumption should be measured at least weekly and if possible daily for water consumption. These data are especially useful for those studies where the test chemical is administered by dosed-feed or dosed-water. Not only are the data necessary for dose calculations, they also provide an excellent indication of the effect of the chemical on the palatability of the food and water and indicate the general health status of the test animals.

Pathology

The gross and microscopic examination of test animal tissues and organs is an extremely important aspect of data collection on a subchronic dose-level-setting study. Complete necropsies should be performed on all animals (Sontag, Page, and Saffiotti, 1976). These necropsies should be performed under the supervision of a qualified pathologist, preferably board-certified. As a minimum, samples of the approximately 30 organs or tissues listed in the *Guidelines for Carcinogen Bioassay in Small Rodents* should be preserved (Sontag, Page, and Saffiotti, 1976). In addition to a careful examination of all internal organs and tissues prior to their removal from the animal, a gross necropsy should include an external examination of all body surfaces and orifices.

A brief review of the literature indicates that there is general agreement concerning the gross observations recommended above (Peck, 1968; World Health Organization, 1978). The recommendations from the microscopic examination vary widely, however, ranging from none (Barnes and Denz, 1954) to tissues from all control animals, the highest dose-level animals without mortality, and the next higher dose-level group (Sontag, Page, and Saffiotti, 1976). In practice, results of subchronic toxicity studies performed with token microscopic examinations compared to those with extensive examinations indicate that the selections of dose levels for chronic studies were more accurate when a reasonable amount of histopathology was available (National Institutes of Health, 1977; National Institutes of Health, 1978; Prejean, 1979).

Other Data

Recently, consideration has been given to the addition of biochemical tests to subchronic dose-level-setting studies. In general, it is felt that these tests could be extremely valuable in evaluating specific toxic effects of selected chemicals; however, for the most part, the literature does not specify a standard set of tests but recommends the inclusion of hematology, clinical chemistry, and pharmacokinetic and behavioral studies for each chemical.

CONCLUSIONS

In reviewing the general parameters for a subchronic toxicity test designed to establish dose levels for a chronic carcinogenicity study, the degree of similarity between this type of study and one designed as an end result study is remarkable. Both types require characterization of the test chemical, an adequate number of test dose levels (usually five), the selection of route based on exposure data in man, exposure over a 90-day period, and the collection of pertinent data such as clinical and gross observations and histopathologic evaluations. Although the use of at least two species is recommended for both types of studies, the end result test has usually relied on a rodent and a nonrodent, whereas the dose-level-setting test has used two species of rodents because of the economic considerations of the long-term study that will follow. The only other major area of disagreement has been in the use of biochemical tests. End result studies have relied on these tests for several years, but it has been only recently that dose-level-setting studies have also begun to take advantage of the data available in selected hematologic or clinical chemistry tests. Thus, unless the current trend changes, it appears that, except for the species difference, which will probably always remain, end result and dose-level-setting subchronic toxicity tests will be virtually identical in the next few years.

REFERENCES FOR APPENDIX A

Barnes, J. M., and F. A. Denz. 1954. Experimental Methods Used in Determining Chronic Toxicity. Pharmcol. Rev. 6:191-242.

Boyland, E., and K. Sydnor. 1962. The Induction of Mammary Cancer in Rats. Br. J. Cancer 16:731-739.

Dunning, W., M. Curtis, and M. Madsen. 1947. Induction of Neoplasms in Five Strains of Rats with Acetylaminofluorene. Cancer Res. 7:134-140.

Federal Register. 1978. Nonclinical Laboratory Studies: Good Laboratory Practice Regulations. 43Fr(247):59986-60025.

McNamara, B. 1976. Concepts in Health Evaluation of Commercial and Industrial Chemicals. In: Advances in Modern Toxicology, Vol. 1, Part 1: New Concepts in Safety Evaluation, M. A. Mehlman, R. E. Shapiro, and H. Blumenthal, eds. John Wiley and Sons, New York. pp. 61-140.

National Academy of Sciences. 1975. Acute and Subchronic Toxicity. In: Principles for Evaluating Chemicals in the Environment. National Academy of Sciences, Washington, D.C. pp. 103-104, 327-330.

National Cancer Institute. 1979. Carcinogenicity Studies in Rodents. National Institutes of Health. RFP No. N01 CP 95619-62. Bethesda, Maryland.

National Institutes of Health. 1977. Bioassay of Tolbutamide for Possible Carcinogenicity. National Cancer Institute Carcinogenesis Technical Report Series No. 47. Department of Health, Education, and Welfare Publication No. (NIH) 77-831. Bethesda, Maryland. 83 pp.

National Institutes of Health. 1978. Bioassay of 4,4'-Thiodianiline for Possible Carcinogenicity. National Cancer Institute Carcinogenesis Technical Report Series No. 47. Department of Health, Education, and Welfare Publication No. (NIH) 78-847. Bethesda, Maryland. 106 pp.

Page, N. 1977. Chronic Toxicity and Carcinogenicity Guidelines. J. Environ. Pathol. Toxicol. 1:161-182.

Peck, H. 1968. An Appraisal of Drug Safety Evaluation in Animals and the Extrapolation of Results to Man. In: Importance of Fundamental Principles in Drug Evaluation, D. H. Tedeschi and R. E. Tedeschi, eds. Raven Press, New York. pp. 450-471.

Pietra, G., H. Rappaport, and P. Shubik. 1961. The Effects of Carcinogenic Chemicals in Newborn Mice. Cancer 14:308-317.

Prejean, J. 1979. Unpublished data. Southern Research Institute. Birmingham, Alabama.

Shimkin, M., S. Smith, P. Shimkin, and H. Andervont. 1962. J. Natl. Cancer Inst. 28:1219.

Smith, C. 1950. A Short-Term Chronic Toxicity Test. J. Pharmcol. Exp. Ther. 100:408-420.

Sontag, J., N. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. Department of Health, Education, and Welfare Publication No. (NIH) 76-801. National Cancer Institute, Bethesda, Maryland. 65 pp.

Weil, C., and D. McCollister. 1963. Relationship Between Short- and Long-Term Feeding Studies in Designing an Effective Toxicity Test. J. Agric. Food Chem. 11(1):486-491.

Weisburger, J., and E. Weisburger. 1967. Tests for Chemical Carcinogens. In: Methods in Cancer Research, Vol. I, H. Busch, ed. Academic Press, New York. pp. 307-398.

World Health Organization. 1978. Acute, Subacute, and Chronic Toxicity Tests. In: Principles and Methods for Evaluating the Toxicity of Chemicals. Geneva. pp. 95-115.

Appendix B

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Appendix B. Chemicals surveyed for Table 4.35

Name[a]	Primary use[b]	Reference
Carbon tetrachloride	Industrial chemical	Adams et al., 1952
3',4'-Dichloropropion anilide	Herbicide	Ambrose et al., 1972
Tetrahydrothiophene-1,1-dioxide; Sulfolane	Solvent	Andersen et al., 1977
Calcium carbimide	Antialcoholic drug	Benitz, Kramer, and Dambach, 1965
1,2,4-Trichlorobenzene	Solvent	Coate et al., 1977
Acrolein; acrylaldehyde, 2-propenal	Cigarette smoke component	Feron et al., 1978; Lyon et al., 1970
Ethylenebisisothiocyanate sulfide	Fungicide	Freudenthal et al., 1977
Triphenyl tin hydroxide; fentin hydroxide	Insect reproduction inhibitor	Gaines and Kimbrough, 1968
Isooctyl isodecyl nylonate	Plasticizer	Gaunt et al., 1969
Ponceau 4R; trisodium salt of 1-(4-sulpho-1-naphthylazo)-2-naphthol-6,8-disulphonic acid	Food color	Gaunt et al., 1967
Orange G; disodium salt of 1-phenylazo-2-naphthol-6,8-disulphonic acid	Food color	Gaunt et al., 1971
Hexachlorobenzene; HCB	Fungicide and industrial by-product	Gralla et al., 1977; Iatropoulos et al., 1978; Iatropoulos et al., 1976; Kimbrough and Linder, 1974; Kuiper-Goodman et al., 1977
Clindamycin hydrochloride; Cleocin	Antibiotic drug	Gray et al., 1972
Triethyl phosphate	Whipping agent	Gumbman, Gagne, and Williams, 1968
Ponceau MX; disodium salt of 1-(2,4-xylylazo)-2-naphthol-3,6-disulphonic acid	Food color	Hall, Lee, and Fairweather, 1966
Dibutyl(diethylene glycol bisphthalate); DDGB	Plasticizer	Hall, Austin, and Fairweather, 1966
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; TCDD	Impurity in pesticides	Harris et al., 1973; Kociba et al., 1976; Zinkl et al., 1973
Quanethidine	Antihypertensive drug	Hartnagel et al., 1976
1-Methyl-3-keto-4-phenylquinuclidinium bromide; MA 540	Antihypertensive drug	Hartnagel et al., 1976
TR2379; trans isomer of N-(1,3,4,6,7-hexahydro-11bH-benzo[*a*]quinolizin-2-yl)propionanilide hydrochloride	Antihypertensive drug	Hartnagel et al., 1975
N-Methyl-*N*-(1-naphthyl)fluoroacetamide; Nissol; MNFA	Pesticide	Hashimoto et al., 1968
2-Methyl-4-chlorophenoxy acetic acid; MCPA	Herbicide	Hattula et al., 1977; Verschuuren, Kroes, and Den Tonkelaar, 1975
2,5,4'-Trichlorobiphenyl	Transformer dielectric fluid	Iatropoulos et al., 1977
Fenterol-HBr; 1-(3,5-dihydroxy-phenyl)-2-{[1-(4-hydroxybenzyl)-ethyl]-amino} ethanol hydrobromide	Beta-sympathomimetic drug	Kast et al., 1975*a*

Appendix B (continued)

Name[a]	Primary use[b]	Reference
Fominoben-HCl; 3'-chloro-2'-[N-methyl-[(morpholino-carbonyl)methyl]-aminomethyl]benzanilide-hydrochloride	Antitussive drug	Kast et al., 1975*b*
Mycotoxin produced by *Aspergillus fumigatus* I21	Animal feed impurity	Khor et al., 1976
Peroxyacetyl nitrate; PAN	Photochemical product in smog	Kruysse et al., 1977
Epichlorohydrin; 1-chloro-2,3-epoxypropane	Epoxy resin	Lawrence et al., 1972
Ferric dimethyl dithiocarbamate; Ferbam	Fungicide	Lee, Russell, and Minor, 1978
Tetramethylthiuram disulfide; Thiram	Fungicide	Lee, Russell, and Minor, 1978
AHR-2438B; sodium salt of a lignosulphonate	Animal feed impurity	Luscombe and Nicholls, 1973
Barthrin; 6-chloropiperonyl ester of chrysanthemumic acid	Insecticide	Masri et al., 1964
Dimethrin; 2,4-dimethylbenzyl ester of chrysanthemumic acid	Insecticide	Masri et al., 1964
Hexachlorophene; 2,2'-methylenebis (3,4,6-trichlorophenol)	Antibacterial and antifungicidal agent	Nakaue, Dost, and Buhler, 1973
Pyridoxine hydrochloride	Antischizophrenic drug	Phillips et al., 1978
Δ^9-Tetrahydrocannabinol; Δ^9-THC	Natural ingredient in marijuana plant	Thompson et al., 1974
2-Methyl-4-chlorophenoxy propionic acid; MCPP	Herbicide	Verschuuren, Kroes, and Den Tonkelaar, 1975

[a] First name listed is the one used in Appendix C.

[b] Primary use as listed by the reference source.

REFERENCES FOR APPENDIX B

Adams, E. M., H. C. Spencer, V. K. Rowe, D. D. McCollister, and D. D. Irish. 1952. Vapor Toxicity of Carbon Tetrachloride Determined by Experiments on Laboratory Animals. Ind. Hyg. Occup. Med. 6:50-66.

Ambrose, A. M., P. S. Larson, J. F. Borzelleca, and R. G. Hennigar, Jr. 1972. Toxicologic Studies on 3',4'-Dichloropropionanilide. Toxicol. Appl. Pharmacol. 23:650-659.

Anderson, M. E., R. A. Jones, R. G. Mehl, T. A. Hill, L. Kurlansik, and L. J. Jenkins, Jr. 1977. The Inhalation Toxicity of Sulfolane (Tetrahydrothiophene-1,1-Dioxide). Toxicol. Appl. Pharmacol. 40:463-472.

Benitz, K. F., A. W. Kramer, Jr., and G. Dambach. 1965. Comparative Studies on the Morphologic Effects of Calcium Carbimide, Propylthiouracil, and Disulfiram in Male Rats. Toxicol. Appl. Pharmacol. 7:128-162.

Coate, W. B., W. H. Schoenfisch, T. R. Lewis, and W. M. Busey. 1977. Chronic Inhalation Exposure of Rats, Rabbits, and Monkeys to 1,2,4-Trichlorobenzene. Arch. Environ. Health 32:249-255.

Cornish, H. H., and R. Hartung. 1969. The Subacute Toxicity of 1,1-Dimethylhydrazine. Toxicol. Appl. Pharmacol. 15:62-68.

Feron, V. J., A. Kruysse, H. P. Til, and H. R. Immel. 1978. Repeated Exposure to Acrolein Vapor: Subacute Studies in Hamsters, Rats, and Rabbits. Toxicology 9:47-57.

Freudenthal, R. I., G. A. Kerchner, R. L. Persing, I. Baumel, and R. L. Baron. 1977. Subacute Toxicity of Ethylenebisisothiocyanate Sulfide in the Laboratory Rat. J. Toxicol. Environ. Health 2:1067-1078.

Gaines, T. B., and R. D. Kimbrough. 1968. Toxicity of Fentin Hydroxide to Rats. Toxicol. Appl. Pharmacol. 12:397-403.

Gaunt, I. F., J. Colley, P. Grasso, M. Creasey, and S. D. Gangolli. 1969. Acute (Rat and Mouse) and Short-Term (Rat) Toxicity Studies on Isooctyl Isodecyl Nylonate. Food Cosmet. Toxicol. 7:115-124.

Gaunt, I. F., M. Farmer, P. Grasso, and S. D. Gangolli. 1967. Acute (Mouse and Rat) and Short-Term (Rat) Toxicity Studies on Ponceau 4R. Food Cosmet. Toxicol. 5:187-194.

Gaunt, I. F., M. Wright, P. Grasso, and S. D. Gangolli. 1971. Short-Term Toxicity of Orange G in Rats. Food Cosmet. Toxicol. 9:329-342.

Gralla, E. J., R. W. Fleischman, Y. K. Luthra, M. Hagopian, J. R. Baker, H. Esber, and W. Marcus. 1977. Toxic Effects of Hexachlorobenzene After Daily Administration to Beagle Dogs for One Year. Toxicol. Appl. Pharmacol. 40:227-239.

Gray, J. E., R. N. Weaver, J. A. Bollert, and E. S. Feenstra. 1972. The Oral Toxicity of Clindamycin in Laboratory Animals. Toxicol. Appl. Pharmacol. 21:516-531.

Gumbmann M. R., W. E. Gagne, and S. N. Williams. 1968. Short-Term Toxicity Studies of Rats Fed Triethyl Phosphate in the Diet. Toxicol. Appl. Pharmacol. 12:360-371.

Hall, D. E., F. S. Lee, and F. A. Fairweather. 1966. Acute (Mouse and Rat) and Short-Term (Rat) Toxicity Studies on Ponceau MX. Food Cosmet. Toxicol. 4:375-382.

Hall, D. E., P. Austin, and F. A. Fairweather. 1966. Acute (Mouse and Rat) and Short-Term (Rat) Toxicity Studies on Dibutyl (Diethylene Glycol Bisphthalate). Food Cosmet. Toxicol. 4:383-388.

Harris, M. W., J. A. Moore, J. G. Vos, and B. N. Gupta. 1973. General Biological Effects of TCDD in Laboratory Animals. Environ. Health Perspect. 5:101-109.

Hartnagel, R. E., B. M. Phillips, E. H. Fonseca, and R. L. Kowalski. 1976. The Acute and Target Organ Toxicity of 1-Methyl-3-Keto-4-Phenylquinuclidinium Bromide (MA 540) and Guanethidine in the Rat and Dog. Arzneim.-Forsch. 26:1671-1672.

Hartnagel, R. E., B. M. Phillips, P. J. Kraus, R. L. Kowalski, and E. H. Fonseca. 1975. A Subchronic Study of the Toxicity of an Orally Administered Benzoquinolizinyl Derivative in the Rat and Dog. Toxicology 4:215-222.

Hashimoto, Y., T. Makita, H. Miyata, T. Noguchi, and G. Ohta. 1968. Acute and Subchronic Toxicity of a New Fluorine Pesticide. *N*-Methyl-*N*-(1-Naphthyl)fluoroacetamide. Toxicol. Appl. Pharmacol. 12:536-547.

Hattula, M. L., H. Elo, H. Reunanen, A. U. Arstila, and T. E. Sorvari. 1977. Acute and Subchronic Toxicity of 2-Methyl-4-Chlorophenoxyacetic Acid (MCPA) in Male Rat. I. Light Microscopy and Tissue Concentrations of MCPA. Bull. Environ. Contam. Toxicol. 18:152-158.

Hunter, C. G., and D. E. Stevenson. 1967. Acute and Subacute Oral Toxicity of Alromine RU 100 in Rats. Food Cosmet. Toxicol. 5:491-496.

Iatropoulos, M. J., J. Bailey, H. P. Adams, F. Coulston, and W. Hobson. 1978. Response of Nursing Infant Rhesus to Clophen A-30 or Hexachlorobenzene Given to Their Lactating Mothers. Environ. Res. 16:38-47.

Iatropoulos, M. J., G. R. Felt, H. P. Adams, F. Korte, and F. Coulston. 1977. Chronic Toxicity of 2,5,4'-Trichlorobiphenyl in Young Rhesus Monkeys. II. Histopathology. Toxicol. Appl. Pharmacol. 41:629-638.

Iatropoulos, M. J., W. Hobson, V. Knauf, and H. P. Adams. 1976. Morphological Effects of Hexachlorobenzine Toxicity in Female Rhesus Monkeys. Toxicol. Appl. Pharmacol. 37:433-444.

Kast, A., Y. Tsunenari, M. Honma, J. Nishikawa, T. Shibata, and M. Torii. 1975*a*. Acute, Subacute, and Chronic Toxicity Studies of the Beta-Sympathomimetic, Fenterol-HBr on Rats, Mice, and Rabbits. Oyo Yakuri. Sendai 10(1):45-71.

Kast, A., Y. Tsunenari, M. Honma, J. Nishikawa, T. Shibata, and M. Torii. 1975*b*. Acute, Subacute, and Chronic Toxicity Studies of an Amino-Halogen-Substituted Benzylamine (Fominoben) in Rats and Mice. Oyo Yakuri. Sendai 10(1):31-43.

Khor, G. L., J. C. Alexander, J. H. Lumsden, and G. J. Losos. 1976. Safety Evaluation of *Aspergillus fumigatus* Grown on Cassava for Use as an Animal Feed. Can. J. Comp. Med. 41:428-434.

Kimbrough, R. D., and R. E. Linder. 1974. The Toxicity of Technical Hexachlorobenzene in the Sherman Strain Rat. A Preliminary Study. Res. Commun. Chem. Pathol. Pharmacol. 8(4):653-664.

Kociba, R. J., P. A. Keeler, C. N. Park, and P. J. Gehrin. 1976. 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD): Results of a 13-Week Oral Toxicity Study in Rats. Toxicol. Appl. Pharmacol. 35:553-574.

Kruysse, A., V. J. Feron, H. R. Immel, B. J. Spit, and G. J. Van Esch. 1977. Short-Term Inhalation Toxicity Studies with Peroxyacetyl Nitrate in Rats. Toxicology 8:231-249.

Kuiper-Goodman, T., D. L. Grant, C. A. Moodie, G. O. Korsrud, and I. C. Munro. 1977. Subacute Toxicity of Hexachlorobenzene in the Rat. Toxicol. Appl. Pharmacol. 40:529-549.

Lawrence, W. H., M. Malik, J. E. Turner, and J. Autian. 1972. Toxicity Profile of Epichlorohydrin. J. Pharmacol. Sci. 61(11):1712-1717.

Lee, C-C., J. Q. Russell, and J. L. Minor. 1978. Oral Toxicity of Ferric Dimethyl Dithiocarbamate (Ferbam) and Tetramethylthiuram Disulfide (Thiram) in Rodents. J. Toxicol. Environ. Health 4:93-106.

Luscombe, D. K., and P. J. Nicholls. 1973. Acute and Subacute Oral Toxicity of AHR-2438B, a Purified Lignosulphonate, in Rats. Food Cosmet. Toxicol. 11:229-237.

Lyon, J. P., L. J. Jenkins, Jr., R. A. Jones, R. A. Coon, and J. Siegel. 1970. Repeated and Continuous Exposure of Laboratory Animals to Acrolein. Toxicol. Appl. Pharmacol. 17:726-732.

Masri, M. S., A. P. Hendrickson, A. J. Cox, Jr., and F. DeEds. 1964. Subacute Toxicity of Two Chrysanthemumic Acid Esters: Barthrin and Dimethrin. Toxicol. Appl. Pharmacol. 6:716-725.

Nakaue, H. S., F. N. Dost, and D. R. Buhler. 1973. Studies on the Toxicity of Hexachlorophene in the Rat. Toxicol. Appl. Pharmacol. 24:239-249.

Phillips, W.E.J., J.H.L. Mills, S. M. Charbonneau, L. Tryphonas, G. V. Hatina, Z. Zawidzka, F. R. Bryce, and I. C. Munro. 1978. Subacute Toxicity of Pyridoxine Hydrochloride in the Beagle Dog. Toxicol. Appl. Pharmacol. 44:323-333.

Thompson, G. R., R. W. Fleischmann, H. Rosenkrantz, and M. C. Braude. 1974. Oral and Intravenous Toxicity of Δ^9-Tetrahydrocannabinol in Rhesus Monkeys. Toxicol. Appl. Pharmacol. 27:648-665.

Verschuuren, H. G., R. Kroes, and E. M. Den Tonkelaar. 1975. Short-Term Oral and Dermal Toxicity of MCPA and MCPP. Toxicology 3:349-359.

Zinkl, J. C., J. G. Vos, J. A. Moore, and B. N. Gupta. 1973. Hematologic and Clinical Chemistry Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Laboratory Animals. Environ. Health Perspect. 5:111-118.

Appendix C

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Appendix C. Species, route of exposure, chemical, and reference data for Table 4.35

Chemical	Species	Route	Result	Reference
Adrenal				
Carbon tetrachloride	Rat	Inhalation	-	1
3,',4'-Dichloropropionanilide	Rat	Oral	-	2
Calcium carbimide	Rat	Oral	+	4
Acrolein	Rat	Inhalation	+	6
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Triphenyl tin hydroxide	Rat	Oral	-	8
Ponceau 4R	Rat	Oral	-	10
Isooctyl isodecyl nylonate	Rat	Oral	+	9
Orange G	Rat	Oral	+	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
	Rat	Oral	+	28
	Rat	Oral	+	31
Clindamycin hydrochloride	Rat	Oral	-	13
	Dog	Oral	-	13
Triethyl phosphate	Rat	Oral	+	14
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	-	17
	Rat	Oral	-	29
TR2379	Rat	Oral	+	19
	Dog	Oral	+	19
Quanethidine	Dog	Oral	-	18
	Rat	Oral	-	18
1-Methyl-3-keto-4-phenylquinuclidinium bromide	Dog	Oral	-	18
	Rat	Oral	-	18
N-Methyl-*N*-(1-naphthyl)fluoracetamide	Rat	Oral	-	20
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	+	21
	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
Fenterol-HBr	Rat	Oral	+	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	-	27
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Epichlorohydrin	Rat	Injection	-	32
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
Ponceau MX	Rat	Oral	-	15
Dibutyl(diethylene glycol bisphthalate)	Rat	Oral	-	16
AHR-2438B	Rat	Oral	-	34
Acrolein	Dog	Inhalation	-	35
Barthrin	Rat	Oral	-	36
Dimethrin	Rat	Oral	-	36
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Injection	-	39
	Monkey	Oral	+	39

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Aorta				
Tetrahydrothiophene-1,1-dioxide	Rat	Inhalation	-	3
	Dog	Inhalation	-	3
	Monkey	Inhalation	-	3
Acrolein	Rat	Inhalation	-	6
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Ponceau 4R	Rat	Oral	-	10
Clindamycin hydrochloride	Dog	Oral	-	13
	Rat	Oral	-	13
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
Fominoben-HCl	Rat	Oral	-	26
Fenterol-HBr	Rat	Oral	-	25
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	-	29
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Δ^9-Tetrahydrocannabinol	Monkey	Injection	-	39
	Monkey	Oral	-	39
Bone				
Calcium carbimide	Rat	Oral	+	4
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
TR2379	Dog	Oral	-	19
	Rat	Oral	-	19
Quanethidine	Rat	Oral	-	18
	Dog	Oral	-	18
1-Methyl-3-keto-4-phenylquinuclidinium bromide	Rat	Oral	-	18
	Dog	Oral	-	18
Hexachlorobenzene	Monkey	Oral	-	24
	Monkey	Oral	-	22
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
Bone Marrow				
Carbon tetrachloride	Rat	Inhalation	-	1
3',4'-Dichloropropionanilide	Rat	Oral	-	2
Calcium carbimide	Rat	Oral	+	4
1,2,4-Trichlorobenzene	Rat	Inhalation	-	5
	Monkey	Inhalation	-	5
Hexachlorobenzene	Dog	Oral	-	12
Clindamycin hydrochloride	Dog	Oral	-	13
	Rat	Oral	-	13
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
N-Methyl-*N*-(1-naphthyl)fluoracetamide	Rat	Oral	-	20
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	-	27

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
Ponceau MX	Rat	Oral	-	15
Dibutyl(diethylene glycol bisphthalate)	Rat	Oral	-	16
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	+	39
	Monkey	Injection	-	39
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
	Brain			
Carbon tetrachloride	Rat	Inhalation	-	1
3',4'-Dichloropropionanilide	Rat	Oral	-	2
Tetrahydrothiophene-1,1-dioxide	Monkey	Inhalation	-	3
	Dog	Inhalation	-	3
	Rat	Inhalation	-	3
Calcium carbimide	Rat	Oral	-	4
1,2,4-Trichlorobenzene	Rat	Inhalation	-	5
	Monkey	Inhalation	-	5
Acrolein	Rat	Inhalation	-	6
	Dog	Inhalation	-	35
	Monkey	Inhalation	-	35
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Triphenyl tin hydroxide	Rat	Oral	-	8
Ponceau 4R	Rat	Oral	-	9
Isooctyl isodecyl nylonate	Rat	Oral	-	10
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	+	22
	Monkey	Oral	+	24
	Rat	Oral	-	28
	Rat	Oral	-	31
Clindamycin hydrochloride	Rat	Oral	-	13
	Dog	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
N-Methyl-*N*-(1-naphthyl)fluoracetamide	Rat	Oral	-	20
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	+	21
	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	+	29
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Epichlorohydrin	Rat	Injection	-	32
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Ponceau MX	Rat	Oral	-	15
Dibutyl(diethylene glycol bisphthalate)	Rat	Oral	-	16
AHR-2438B	Rat	Oral	-	34
Hexachlorophene	Rat	Oral	+	37
Pyridoxine hydrochloride	Dog	Oral	+	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2,5,4'-Trichlorobiphenyl	Monkey	Oral	+	23
Esophagus				
Tetrahydrothiophene-1,1-dioxide	Monkey	Inhalation	-	3
	Dog	Inhalation	-	3
	Rat	Inhalation	-	3
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Clindamycin hydrochloride	Dog	Oral	-	13
	Rat	Oral	-	13
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
Hexachlorobenzene	Monkey	Oral	-	22
	Monkey	Oral	-	24
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	-	29
AHR-2438B	Rat	Oral	-	34
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
Eye				
1,2,4-Trichlorobenzene	Rat	Inhalation	-	5
	Monkey	Inhalation	-	5
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
2,5,4'-Trichlorophenyl	Monkey	Oral	-	23
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	-	29
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
Gallbladder				
Tetrahydrothiophene-1,1-dioxide	Monkey	Inhalation	-	3
	Dog	Inhalation	-	3
	Rat	Inhalation	-	3
Hexachlorobenzene	Dog	Oral	-	12
Clindamycin hydrochloride	Dog	Oral	+	13
	Rat	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
Δ^9-Tetrahydracannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39

Appendix C (continued)

Chemical	Species	Route	Result	Reference
	Heart			
Carbon tetrachloride	Rat	Inhalation	-	1
3',4'-Dichloropropionanilide	Rat	Oral	+	2
Tetrahydrothiophene-1,1-dioxide	Rat	Inhalation	-	3
	Dog	Inhalation	-	3
	Monkey	Inhalation	+	3
Calcium carbimide	Rat	Oral	-	4
1,2,4-Trichlorobenzene	Monkey	Inhalation	-	5
	Rat	Inhalation	-	5
Acrolein	Rat	Inhalation	+	6
	Rat	Inhalation	-	35
	Dog	Inhalation	-	35
	Monkey	Inhalation	-	35
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Triphenyl tin hydroxide	Rat	Oral	-	8
Ponceau 4R	Rat	Oral	-	10
Isooctyl isodecyl nitrate	Rat	Oral	-	9
Orange G	Rat	Oral	+	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
	Rat	Oral	+	28
	Rat	Oral	-	31
Clindamycin hydrochloride	Rat	Oral	-	13
	Dog	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	-	17
	Rat	Oral	-	29
TR2379	Rat	Oral	+	19
	Dog	Oral	+	19
Quanethidine	Dog	Oral	-	18
	Rat	Oral	-	18
1-Methyl-3-keto-4-phenylquinuclidinium bromide	Dog	Oral	-	18
	Rat	Oral	-	18
N-Methyl-*N*-(1-naphthyl)fluoracetamide	Rat	Oral	-	20
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	21
	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
Fenterol-HBr	Rat	Oral	+	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	-	27
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Epichlorohydrin	Rat	Injection	-	32
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	34
Ponceau MX	Rat	Oral	-	15
Dibutyl(diethylene glycol bisphthalate)	Rat	Oral	-	16
AHR-2438B	Rat	Oral	-	34
Barthrin	Rat	Oral	-	36

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Dimethrin	Rat	Oral	-	36
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
	Kidney			
Carbon tetrachloride	Rat	Inhalation	+	1
3',4'-Dichloropropionanilide	Rat	Oral	+	2
Tetrahydrothiophene-1,1-dioxide	Rat	Inhalation	-	3
	Monkey	Inhalation	-	3
	Dog	Inhalation	-	3
Calcium carbimide	Rat	Oral	-	4
1,2,4-Trichlorobenzene	Rat	Inhalation	+	5
	Monkey	Inhalation	-	5
Acrolein	Rat	Inhalation	+	6
	Rat	Inhalation	+	35
	Monkey	Inhalation	+	35
	Dog	Inhalation	+	35
Ethylenebisisothiocyanate sulfide	Rat	Oral	+	7
Triphenyl tin hydroxide	Rat	Oral	+	8
Ponceau 4R	Rat	Oral	-	10
Isooctyl isodecyl nylonate	Rat	Oral	+	9
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Dog	Oral	+	12
	Monkey	Oral	+	22
	Monkey	Oral	+	24
	Rat	Oral	+	28
	Rat	Oral	+	31
Clindamycin hydrochloride	Rat	Oral	+	13
	Dog	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	-	17
	Rat	Oral	-	29
TR2379	Rat	Oral	+	19
	Dog	Oral	+	19
Quanethidine	Dog	Oral	-	18
	Rat	Oral	-	18
1-Methyl-3-keto-4-phenylquinuclidinium bromide	Dog	Oral	-	18
	Rat	Oral	-	18
N-Methyl-*N*-(1-naphthyl)fluoractemide	Rat	Oral	+	20
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	+	21
	Rat	Oral	+	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	+	40
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	+	27
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Epichlorohydrin	Rat	Injection	+	32
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Tetramethylthiuram disulfide	Rat	Oral	-	33
Ponceau MX	Rat	Oral	+	15
Dibutyl(diethylene glycol bisphthalate)	Rat	Oral	-	16
AHR-2438B	Rat	Oral	+	34
Barthrin	Rat	Oral	+	36
Dimethrin	Rat	Oral	+	36
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2,5,4'-Trichlorobiphenyl	Monkey	Oral	+	23
Large Intestine				
Carbon tetrachloride	Rat	Inhalation	-	1
3',4'-Dichloropropionanilide	Rat	Oral	-	2
Tetrahydrothiophene-1,1-dioxide	Rat	Inhalation	-	3
	Dog	Inhalation	-	3
	Monkey	Inhalation	-	3
Acrolein	Rat	Inhalation	-	6
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
Clindamycin hydrochloride	Dog	Oral	-	13
	Rat	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
Quanethidine	Dog	Oral	-	18
	Rat	Oral	-	18
1-Methyl-3-keto-4-phenylquinuclidinium bromide	Dog	Oral	-	18
	Rat	Oral	-	18
N-Methyl-*N*-(1-naphthyl)fluoracetamide	Rat	Oral	+	20
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	+	26
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	-	29
Peroxyacetyl nitrate	Rat	Injection	-	30
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
AHR-2438B	Rat	Oral	-	34
Barthrin	Rat	Oral	-	36
Dimethrin	Rat	Oral	-	36
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	+	39

Appendix C (continued)

Chemical	Species	Route	Result	Reference
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
	Liver			
Carbon tetrachloride	Rat	Inhalation	+	1
3',4'-Dichloropropionanilide	Rat	Oral	+	2
Tetrahydrothiophene-1,1-dioxide	Rat	Inhalation	-	3
	Dog	Inhalation	-	3
	Monkey	Inhalation	+	3
Calcium carbimide	Rat	Oral	+	4
1,2,4-Trichlorobenzene	Rat	Inhalation	+	5
	Monkey	Inhalation	-	5
Acrolein	Rat	Inhalation	-	6
	Rat	Inhalation	+	35
	Dog	Inhalation	+	35
	Monkey	Inhalation	+	35
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Triphenyl tin hydroxide	Rat	Oral	-	8
Isooctyl isodecyl nylonate	Rat	Oral	+	9
Ponceau 4R	Rat	Oral	-	10
Orange G	Rat	Oral	+	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	+	24
	Rat	Oral	+	28
	Rat	Oral	+	31
Clindamycin hydrochloride	Rat	Oral	-	13
	Dog	Oral	-	13
Triethyl phosphate	Rat	Oral	+	14
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	+	17
	Rat	Oral	+	29
TR2379	Rat	Oral	+	19
	Dog	Oral	+	19
Quanethidine	Rat	Oral	-	18
	Dog	Oral	-	18
1-Methyl-3-keto-4-phenylquinuclidinium bromide	Dog	Oral	-	18
	Rat	Oral	-	18
N-Methyl-*N*-(1-naphthyl)fluoracetamide	Rat	Oral	-	20
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	+	21
	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
Fenterol-HBr	Rat	Oral	+	25
Fominoben-HCl	Rat	Oral	+	26
Aspergillus fumigatus	Rat	Oral	-	27
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Epichlorohydrin	Rat	Injection	-	32
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Ponceau MX	Rat	Oral	+	15
Dibutyl(diethylene glycol bisphthalate)	Rat	Oral	-	16
AHR-2438B	Rat	Oral	+	34
Barthrin	Rat	Oral	+	36
Dimethrin	Rat	Oral	+	36
Hexachlorophene	Rat	Oral	-	37
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	+	39
	Monkey	Injection	+	39
2,5,4'-Trichlorobiphenyl	Monkey	Oral	+	23
Lung				
Carbon tetrachloride	Rat	Inhalation	-	1
3',4'-Dichloropropionanilide	Rat	Oral	-	2
Tetrahydrothiophene-1,1-dioxide	Dog	Inhalation	+	3
	Rat	Inhalation	+	3
	Monkey	Inhalation	+	3
Calcium carbimide	Rat	Oral	-	4
1,2,4-Trichlorobenzene	Rat	Inhalation	-	5
	Monkey	Inhalation	-	5
Acrolein	Rat	Inhalation	+	6
	Rat	Inhalation	+	35
	Dog	Inhalation	+	35
	Monkey	Inhalation	+	36
Ethylenebisisothiocyanate sulfide	Rat	Oral	+	7
Triphenyl tin hydroxide	Rat	Oral	+	8
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Orange G	Rat	Oral	-	10
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	24
	Monkey	Oral	+	22
	Rat	Oral	+	28
	Rat	Oral	-	31
Clindamycin hydrochloride	Rat	Oral	-	13
	Dog	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	-	17
	Rat	Oral	-	29
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
Quanethidine	Rat	Oral	-	18
	Dog	Oral	-	18
1-Methyl-3-keto-4-phenylquinuclidinium bromide	Rat	Oral	-	18
	Dog	Oral	-	18
N-Methyl-*N*-(1-naphthyl)fluoracetamide	Rat	Oral	-	20
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	21
	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Aspergillus fumigatus	Rat	Oral	-	27
Peroxyacetyl nitrate	Rat	Inhalation	+	30
Epichlorohydrin	Rat	Injection	+	32
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
AHR-2438B	Rat	Oral	-	34
Barthrin	Rat	Oral	-	36
Dimethrin	Rat	Oral	-	36
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	+	39
2,5,4'-Trichlorobiphenyl	Monkey	Oral	+	23
	Lymph Node			
Carbon tetrachloride	Rat	Inhalation	-	1
Tetrahydrothiophene-1,1-dioxide	Rat	Inhalation	-	3
	Dog	Inhalation	-	3
	Monkey	Inhalation	-	3
Calcium carbimide	Rat	Oral	+	4
Acrolein	Rat	Inhalation	-	6
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Dog	Oral	+	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
Clindamycin hydrochloride	Dog	Oral	-	13
	Rat	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Rat	Oral	+	29
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Ferric dimethyl dithiocarbamate	Rat	Oral	+	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
AHR-2438B	Rat	Oral	+	34
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Mammary Gland				
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
Muscle Tissue				
Carbon tetrachloride	Rat	Inhalation	-	1
3',4'-Dichloropropionanilide	Rat	Oral	-	2
Acrolein	Rat	Inhalation	-	6
Triphenyl tin hydroxide	Rat	Oral	-	8
Orange G	Rat	Oral	-	11
Clindamycin hydrochloride	Dog	Oral	-	13
	Rat	Oral	-	13
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	21
	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Rat	Oral	-	29
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
Nerve Tissue				
Carbon tetrachloride	Rat	Inhalation	-	1
Quanethidine	Dog	Oral	+	18
	Rat	Oral	+	18
1-Methyl-3-keto-4-phenylquinuclidinium bromide	Dog	Oral	-	18
	Rat	Oral	-	18
Hexachlorobenzene	Monkey	Oral	+	22
	Monkey	Oral	+	24
	Rat	Oral	-	28
Aspergillus fumigatus	Rat	Oral	-	27
Pyridoxine hydrochloride	Dog	Oral	+	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
2,5,4'-Trichlorobiphenyl	Monkey	Oral	+	23

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Ovaries				
3',4'-Dichloropropionanilide	Rat	Oral	-	2
Calcium carbimide	Rat	Oral	+	4
Acrolein	Rat	Inhalation	-	6
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Triphenyl tin hydroxide	Rat	Oral	-	8
Ponceau MX	Rat	Oral	-	10
Isooctyl isodecyl nylonate	Rat	Oral	+	9
Orange G	Rat	Oral	+	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	+	24
	Monkey	Oral	-	22
	Rat	Oral	-	31
Clindamycin hydrochloride	Dog	Oral	-	13
	Rat	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
N-Methyl-*N*-(1-naphthyl)fluoracetamide	Rat	Oral	-	20
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	+	29
Epichlorohydrin	Rat	Injection	-	32
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
Ponceau MX	Rat	Oral	-	16
Dibutyl(diethylene glycol bisphthalate)	Rat	Oral	-	17
AHR-2438B	Rat	Oral	-	34
Barthrin	Rat	Oral	-	36
Dimethrin	Rat	Oral	-	36
Hexachlorophene	Rat	Oral	-	37
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	+	40
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
Pancreas				
Carbon tetrachloride	Rat	Inhalation	-	1
3',4'-Dichloropropionanilide	Rat	Oral	-	2
Tetrahydrothiophene-1,1-dioxide	Rat	Inhalation	-	3
	Dog	Inhalation	-	3
	Monkey	Inhalation	-	3
Calcium carbimide	Rat	Oral	-	4
Acrolein	Rat	Inhalation	-	6
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
	Rat	Oral	-	31
Clindamycin hydrochloride	Rat	Oral	-	13
	Dog	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
N-Methyl-*N*-(1-naphthyl)fluoracetamide	Rat	Oral	-	20
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	21
	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
AHR-2438B	Rat	Oral	-	34
Barthrin	Rat	Oral	-	36
Dimethrin	Rat	Oral	-	36
Δ^9-Tetrahydrocannabinol	Monkey	Oral	+	39
	Monkey	Injection	-	39
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
Pituitary				
Carbon tetrachloride	Rat	Oral	-	1
Calcium carbimide	Rat	Oral	+	4
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
Clindamycin hydrochloride	Dog	Oral	-	13
	Rat	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	-	29
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
AHR-2438B	Rat	Oral	-	34
Pyridoxine hydrochloride	Dog	Oral	-	38

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
	Prostate			
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Hexachlorobenzene	Rat	Oral	-	12
Clindamycin hydrochloride	Rat	Oral	-	13
	Dog	Oral	-	13
TR2379	Dog	Oral	-	19
	Rat	Oral	-	19
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	21
	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	+	40
Fenterol-HBr	Rat	Oral	+	25
Fominoben-HCl	Rat	Oral	+	26
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Rat	Oral	-	29
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
	Salivary Gland			
Calcium carbimide	Rat	Oral	-	4
Acrolein	Rat	Inhalation	-	6
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Triphenyl tin hydroxide	Rat	Oral	-	8
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
Clindamycin hydrochloride	Rat	Oral	+	13
	Dog	Oral	-	13
TR2379	Dog	Oral	-	19
	Rat	Oral	-	19
Fenterol-HBr	Rat	Oral	+	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Rat	Oral	-	29
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
AHR-2438B	Rat	Oral	-	34

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
Sciatic Nerve				
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Hexachlorobenzene	Dog	Oral	-	12
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	-	29
Pyridoxine hydrochloride	Dog	Oral	+	38
Skin				
3',4'-Dichloropropionanilide	Rat	Oral	-	2
1,2,4-Trichlorobenzene	Rat	Inhalation	-	5
	Monkey	Inhalation	-	5
Acrolein	Rat	Inhalation	-	6
Hexachlorobenzene	Dog	Oral	-	12
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	-	29
Peroxyacetyl nitrate	Rat	Inhalation	-	30
AHR-2438B	Rat	Oral	+	34
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
Small Intestine				
Carbon tetrachloride	Rat	Inhalation	-	1
3',4'-Dichloropropionanilide	Rat	Oral	-	2
Tetrahydrothiophene-1,1-dioxide	Dog	Inhalation	-	3
	Rat	Inhalation	-	3
	Monkey	Inhalation	-	3
Acrolein	Rat	Inhalation	-	6
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Orange G	Rat	Oral	+	11
Triethyl phosphate	Rat	Oral	-	14
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
Clindamycin hydrochloride	Rat	Oral	-	13
	Dog	Oral	-	13
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
Quanethidine	Dog	Oral	-	18
	Rat	Oral	-	18
1-Methyl-3-keto-4-phenylquinuclidinium bromide	Dog	Oral	-	18
	Rat	Oral	-	18
N-Methyl-*N*-(1-naphthyl)fluoracetamide	Rat	Oral	-	20
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	21
	Rat	Oral	-	40

Appendix C (continued)

Chemical	Species	Route	Result	Reference
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Rat	Oral	-	29
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
AHR-2438B	Rat	Oral	-	34
Barthrin	Rat	Oral	-	36
Dimethrin	Rat	Oral	-	36
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
Spinal Cord				
1,2,4-Trichlorobenzene	Rat	Inhalation	-	5
	Monkey	Inhalation	-	5
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	+	22
	Monkey	Oral	+	24
	Rat	Oral	-	31
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Rat	Oral	-	29
Acrolein	Dog	Inhalation	-	35
	Monkey	Inhalation	-	35
Pyridoxine hydrochloride	Dog	Oral	+	38
2,5,4'-Trichlorobiphenyl	Monkey	Oral	+	23
Spleen				
Carbon tetrachloride	Rat	Inhalation	+	1
3',4'-Dichloropropionanilide	Rat	Oral	+	2
Tetrahydrothiophene-1,1-dioxide	Rat	Inhalation	-	3
	Dog	Inhalation	-	3
	Monkey	Inhalation	-	3
Calcium carbimide	Rat	Oral	+	4
1,2,4-Trichlorobenzene	Rat	Inhalation	-	5
	Monkey	Inhalation	-	5
Acrolein	Rat	Inhalation	-	6
	Rat	Inhalation	-	35
	Dog	Inhalation	-	35
	Monkey	Inhalation	-	35
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Triphenyl tin hydroxide	Rat	Oral	+	8

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Ponceau 4R	Rat	Oral	-	10
Orange G	Rat	Oral	+	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
	Rat	Oral	+	28
	Rat	Oral	+	31
Clindamycin hydrochloride	Rat	Oral	-	13
	Dog	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	-	17
	Rat	Oral	+	29
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
N-Methyl-*N*-(1-naphthyl)fluoracetamide	Rat	Oral	-	20
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	+	21
	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	-	27
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Epichlorohydrin	Rat	Injection	-	32
Ferric dimethyl dithiocarbamate	Rat	Oral	+	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
Ponceau MX	Rat	Oral	+	15
Dibutyl(diethylene glycol bisphthalate)	Rat	Oral	-	16
AHR-2438B	Rat	Oral	+	34
Barthrin	Rat	Oral	-	36
Dimethrin	Rat	Oral	-	36
Hexachlorophene	Rat	Oral	-	37
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
	Stomach			
Carbon tetrachloride	Rat	Inhalation	-	1
3',4'-Dichloropropionanilide	Rat	Oral	-	2
Tetrahydrothiophene-1,1-dioxide	Rat	Inhalation	-	3
	Dog	Inhalation	-	3
	Monkey	Inhalation	-	3
Acrolein	Rat	Inhalation	-	6
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Dog	Oral	+	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Clindamycin hydrochloride	Dog	Oral	+	13
	Rat	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
Quanethidine	Rat	Oral	-	18
	Dog	Oral	-	18
1-Methyl-3-keto-4-phenylquinuclidinium bromide	Rat	Oral	-	18
	Dog	Oral	-	18
N-Methyl-N-(1-naphthyl)fluoracetamide	Rat	Oral	-	20
Fominoben-HCl	Rat	Oral	+	26
Fenterol-HBr	Rat	Oral	-	25
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Rat	Oral	-	29
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
AHR-2438B	Rat	Oral	-	34
Barthrin	Rat	Oral	-	36
Dimethrin	Rat	Oral	-	36
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
	Testes			
Carbon tetrachloride	Rat	Inhalation	+	1
3',4'-Dichloropropionanilide	Rat	Oral	+	2
Calcium carbimide	Rat	Oral	+	4
Acrolein	Rat	Inhalation	-	6
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Triphenyl tin hydroxide	Rat	Oral	+	8
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Ponceau 4R	Rat	Oral	-	10
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Dog	Oral	+	12
	Rat	Oral	-	28
	Rat	Oral	-	31
Clindamycin hydrochloride	Dog	Oral	-	13
	Rat	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Rat	Oral	-	17
	Rat	Oral	+	29
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19

Appendix C (continued)

Chemical	Species	Route	Result	Reference
N-Methyl-*N*-(1-naphthyl)fluoracetamide	Rat	Oral	+	20
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	+	21
	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
Fenterol-HBr	Rat	Oral	+	25
Fominoben-HCl	Rat	Oral	+	26
Aspergillus fumigatus	Rat	Oral	-	27
Peroxyacetyl nitrate	Rat	Inhalation	+	30
Epichlorohydrin	Rat	Injection	-	32
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
Ponceau MX	Rat	Oral	-	15
Dibutyl(diethylene glycol bisphthalate)	Rat	Oral	-	16
AHR-2438B	Rat	Oral	-	34
Barthrin	Rat	Oral	-	36
Dimethrin	Rat	Oral	-	36
Hexachlorophene	Rat	Oral	-	37
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Injection	-	39
	Monkey	Oral	+	39
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
Thymus				
Calcium carbimide	Rat	Oral	-	4
Acrolein	Rat	Inhalation	-	6
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Orange G	Rat	Oral	-	11
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	Rat	Oral	+	17
	Rat	Oral	+	29
Quanethidine	Dog	Oral	-	18
	Rat	Oral	-	18
1-Methyl-3-keto-4-phenylquinuclidinium bromide	Dog	Oral	-	18
	Rat	Oral	-	18
N-Methyl-*N*-(1-naphthyl)fluoracetamide	Rat	Oral	-	20
Hexachlorobenzene	Monkey	Oral	+	24
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	+	26
Aspergillus fumigatus	Rat	Oral	-	27
Peroxyacetyl nitrate	Rat	Inhalation	-	30
TR2379	Rat	Oral	-	34
Δ^9-Tetrahydrocannabinol	Monkey	Oral	+	39
	Monkey	Injection	+	39
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40

Appendix C (continued)

Chemical	Species	Route	Result	Reference
	Thyroid			
3,'4'-Dichloropropionanilide	Rat	Oral	-	2
Tetrahydrothiophene-1,1-dioxide	Rat	Inhalation	-	3
	Dog	Inhalation	-	3
	Monkey	Inhalation	-	3
Calcium carbimide	Rat	Oral	+	4
Acrolein	Rat	Inhalation	-	6
	Dog	Inhalation	-	35
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
	Rat	Oral	-	31
Clindamycin hydrochloride	Rat	Oral	-	13
	Dog	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
TR2379	Rat	Oral	+	19
	Dog	Oral	+	19
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	21
	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	+	26
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Rat	Oral	-	29
Peroxyacetyl nitrate	Rat	Inhalation	+	30
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
AHR-2438B	Rat	Oral	-	34
Barthrin	Rat	Oral	-	36
Dimethrin	Rat	Oral	-	36
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
	Trachea			
Tetrahydrothiophene-1,1-dioxide	Rat	Inhalation	-	3
	Dog	Inhalation	-	3
	Monkey	Inhalation	-	3
Acrolein	Rat	Inhalation	+	6
	Dog	Inhalation	+	35
	Monkey	Inhalation	+	35
Ethylenebisisothiocyanate sulfide	Rat	Oral	+	7
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Monkey	Oral	-	22
	Monkey	Oral	-	24

Appendix C (continued)

Chemical	Species	Route	Result	Reference
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Rat	Oral	-	29
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Δ^9-Tetrahydrocannabinol	Monkey	Injection	-	39
	Monkey	Oral	-	39
Urinary Bladder				
Carbon tetrachloride	Rat	Inhalation	-	1
3',4'-Dichloropropionanilide	Rat	Oral	-	2
Tetrahydrothiophene-1,1-dioxide	Rat	Inhalation	-	3
	Dog	Inhalation	-	3
	Monkey	Inhalation	-	3
Acrolein	Rat	Inhalation	-	6
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
Clindamycin hydrochloride	Dog	Oral	-	13
	Rat	Oral	-	13
Triethyl phosphate	Rat	Oral	-	14
TR2379	Rat	Oral	-	19
	Dog	Oral	-	19
Fenterol-HBr	Rat	Oral	-	25
Fominoben-HCl	Rat	Oral	-	26
Aspergillus fumigatus	Rat	Oral	-	27
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Rat	Oral	-	29
Peroxyacetyl nitrate	Rat	Inhalation	-	30
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
AHR-2438B	Rat	Oral	-	34
Barthrin	Rat	Oral	-	36
Dimethrin	Rat	Oral	-	36
Pyridoxine hydrochloride	Dog	Oral	-	38
Δ^9-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40

Appendix C (continued)

Chemical	Species	Route	Result	Reference
Uterus				
Acrolein	Rat	Inhalation	-	6
Ethylenebisisothiocyanate sulfide	Rat	Oral	-	7
Isooctyl isodecyl nylonate	Rat	Oral	-	9
Orange G	Rat	Oral	-	11
Hexachlorobenzene	Dog	Oral	-	12
	Monkey	Oral	-	22
	Monkey	Oral	-	24
2,5,4'-Trichlorobiphenyl	Monkey	Oral	-	23
Clindamycin hydrochloride	Rat	Oral	-	13
	Dog	Oral	-	13
TF2379	Rat	Oral	-	19
	Dog	Oral	-	19
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Rat	Oral	+	29
Ferric dimethyl dithiocarbamate	Rat	Oral	-	33
Tetramethylthiuram disulfide	Rat	Oral	-	33
Pyridoxine hydrochloride	Dog	Oral	-	38
-Tetrahydrocannabinol	Monkey	Oral	-	39
	Monkey	Injection	-	39
2-Methyl-4-chlorophenoxy acetic acid	Rat	Oral	-	40
2-Methyl-4-chlorophenoxy propionic acid	Rat	Oral	-	40

REFERENCES FOR APPENDIX C

1. Adams, E. M., H. C. Spencer, V. K. Rowe, D. D. McCollister, and D. D. Irish. 1952. Vapor Toxicity of Carbon Tetrachloride Determined by Experiments on Laboratory Animals. Ind. Hyg. Occup. Med. 6:50-66.

2. Ambrose, A. M., P. S. Larson, J. F. Borzelleca, and R. G. Hennigar, Jr. 1972. Toxicologic Studies on 3',4'-Dichloropropionanilide. Toxicol. Appl. Pharmacol. 23:650-659.

3. Anderson, M. E., R. A. Jones, R. G. Mehl, T. A. Hill, L. Kurlansik, and L. J. Jenkins, Jr. 1977. The Inhalation Toxicity of Sulfolane (Tetrahydrothiophene-1,1-Dioxide). Toxicol. Appl. Pharmacol. 40:463-472.

4. Benitz, K. F., A. W. Kramer, Jr., and G. Dambach. 1965. Comparative Studies on the Morphologic Effects of Calcium Carbimide, Propylthiouracil, and Disulfiram in Male Rats. Toxicol. Appl. Pharmacol. 7:128-162.

5. Coate, W. B., W. H. Schoenfisch, T. R. Lewis, and W. M. Busey. 1977. Chronic, Inhalation Exposure of Rats, Rabbits, and Monkeys to 1,2,4-Trichlorobenzene. Arch. Environ. Health 23:249-255.

6. Feron, V. J., A. Kruysse, H. P. Til, and H. R. Immel. 1978. Repeated Exposure to Acrolein Vapour: Subacute Studies in Hamsters, Rats, and Rabbits. Toxicology 9:47-57.

7. Freudenthal, R. I., G. A. Kerchner, R. L. Persing, I. Baumel, and R. L. Baron. 1977. Subacute Toxicity of Ethylenebisisothiocyanate Sulfide in the Laboratory Rat. J. Toxicol. Environ. Health 2:1067-1078.

8. Gaines, T. B., and R. D. Kimbrough. 1968. Toxicity of Fentin Hydroxide to Rats. Toxicol. Appl. Pharmacol. 12:397-403.

9. Gaunt, I. F., J. Colley, P. Grasso, M. Creasey, and S. D. Gangolli. 1969. Acute (Rat and Mouse) and Short-Term (Rat) Toxicity Studies on Isooctyl Isodecyl Nylonate. Food Cosmet. Toxicol. 7:115-124.

10. Gaunt, I. F., M. Farmer, P. Grasso, and S. D. Gangolli. 1967. Acute (Mouse and Rat) and Short-Term (Rat) Toxicity Studies on Ponceau 4R. Food Cosmet. Toxicol. 5:187-194.

11. Gaunt, I. F., M. Wright, P. Grasso, and S. D. Gangolli. 1971. Short-Term Toxicity of Orange G in Rats. Food Cosmet. Toxicol. 9:329-342.

12. Gralla, E. J., R. W. Fleischman, Y. K. Luthra, M. Hagopian, J. R. Baker, H. Esber, and W. Marcus. 1977. Toxic Effects of Hexachlorobenzene After Daily Administration to Beagle Dogs for One Year. Toxicol. Appl. Pharmacol. 40:227-239.

13. Gray, J. E., R. N. Weaver, J. A. Bollert, and E. S. Feenstra. 1972. The Oral Toxicity of Clindamycin in Laboratory Animals. Toxicol. Appl. Pharmacol. 21:516-531.

14. Gumbmann, M. R., W. E. Gagne, and S. N. Williams. 1968. Short-Term Toxicity Studies of Rats Fed Triethyl Phosphate in the Diet. Toxicol. Appl. Pharmacol. 12:360-371.

15. Hall, D. E., F. S. Lee, and F. A. Fairweather. 1966. Acute (Mouse and Rat) and Short-Term (Rat) Toxicity Studies on Ponceau MX. Food Cosmet. Toxicol. 4:375-382.

16. Hall, D. E., P. Austin, and F. A. Fairweather. 1966. Acute (Mouse and Rat) and Short-Term (Rat) Toxicity Studies on Dibutyl (Diethylene Glycol Bisphthalate). Food Cosmet. Toxicol. 4:383-388.

17. Harris, M. W., J. A. Moore, J. G. Vos, and B. N. Gupta. 1973. General Biological Effects of TCDD in Laboratory Animals. Environ. Health Perspect. 5:101-109.

18. Hartnagel, R. E., B. M. Phillips, E. H. Fonseca, and R. L. Kowalski. 1976. The Acute and Target Organ Toxicity of 1-Methyl-3-Keto-4-Phenylquinuclidinium Bromide (MA 540) and Guanethidine in the Rat and Dog. Arzneim.-Forsch. 26:1671-1672.

19. Hartnagel, R. E., B. M. Phillips, P. J. Kraus, R. L. Kowalski, and E. H. Fonseca. 1975. A Subchronic Study of the Toxicity of an Orally Administered Benzoquinolizinyl Derivative in the Rat and Dog. Toxicology. 4:215-222.

20. Hashimoto, Y., T. Makita, H. Miyata, T. Noguchi, and G. Ohta. 1968. Acute and Subchronic Toxicity of a New Fluorine Pesticide, *N*-Methyl-*N*-(1-Naphtyl)fluoracetamide. Toxicol. Appl. Pharmacol. 12:536-547.

21. Hattula, M. L., H. Elo, H. Reunanen, A. U. Arstila, and T. E. Sorvari. 1977. Acute and Subchronic Toxicity of 2-Methyl-4-Chlorophenoxy Acetic Acid (MCPA) in Male Rat. I. Light Microscopy and Tissue Concentrations of MCPA. Bull. Environ. Contam. Toxicol. 18:152-158.

22. Iatropoulos, M. J., J. Bailey, H. P. Adams, F. Coulston, and W. Hobson. 1978. Response of Nursing Infant Rhesus to Clophen A-30 or Hexachlorobenzene Given to Their Lactating Mothers. Environ. Res. 16:38-47.

23. Iatropoulos, M. J., G. R. Felt, H. P. Adams, F. Korte, and F. Coulston. 1977. Chronic Toxicity of 2,5,4'-Trichlorobiphenyl in Young Rhesus Monkeys. II. Histopathology. Toxicol. Appl. Pharmacol. 41:629-638.

24. Iatropoulos, M. J., W. Hobson, V. Knauf, and H. P. Adams. 1976. Morphological Effects of Hexachlorobenzene Toxicity in Female Rhesus Monkeys. Toxicol. Appl. Pharmacol. 37:433-444.

25. Kast, A., Y. Tsunerari, M. Honma, J. Nishikawa, T. Shibata, and M. Torii. 1975*a*. Acute, Subacute, and Chronic Toxicity Studies of the Beta-Sympathomimetic, Fenterol-HBr on Rats, Mice, and Rabbits. Oyo Yakuri. Sendai 10(1):45-71.

26. Kast, A., Y. Tsunenari, M. Honma, J. Nishikawa, T. Shibata, and M. Torii. 1975*b*. Acute, Subacute, and Chronic Toxicity Studies of an Amino-Halogen-Substituted Benzylamine (Fominoben) in Rats and Mice. Oyo Yakuri. Sendai 10(1):31-43.

27. Khor, G. L., J. C. Alexander, J. H. Lumsden, and G. J. Losos. 1976. Safety Evaluation of *Aspergillus fumigatus* Grown on Cassava for Use as an Animal Feed. Can. J. Comp. Med. 41:428-434.

28. Kimbrough, R. D., and R. E. Linder. 1974. The Toxicity of Technical Hexachlorobenzene in the Sherman Strair Rat. A Preliminary Study. Res. Commun. Chem. Pathol. Pharmacol. 8(4):653-664.

29. Kociba, R. J., P. A. Keeler, C. N. Park, and P. J. Gehring. 1976. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD): Results of a 13-Week Oral Toxicity Study in Rats. Toxicol. Appl. Pharmacol. 35:553-574.

30. Kruysse, A., V. J. Feron, H. R. Immel, B. J. Spit, and G. J. Van Esch. 1977. Short-Term Inhalation Toxicity Studies with Peroxyacetyl Nitrate in Rats. Toxicology 8:231-249.

31. Kuiper-Goodman, T., D. L. Grant, C. A. Moodie, G. O. Korsrud, and I. C. Munro. 1977. Subacute Toxicity of Hexachlorobenzene in the Rat. Toxicol. Appl. Pharmacol. 40:529-549.

32. Lawrence, W. H., M. Malik, J. E. Turner, and J. Autian. 1972. Toxicity Profile of Epichlorohydrin. J. Pharmacol. Sci. 61(11): 1712-1717.

33. Lee, C-C., J. Q. Russell, and J. L. Minor. 1978. Oral Toxicity of Ferric Dimethyl Dithiocarbamate (Ferbam) and Tetramethylthiuram Disulfide (Thiram) in Rodents. J. Toxicol. Environ. Health 4:93-106.

34. Luscombe, D. K., and P. J. Nicholls. 1973. Acute and Subacute Oral Toxicity of AHR-2438B, a Purified Lignosulphonate, in Rats. Food Cosmet. Toxicol. 11:229-237.

35. Lyon, J. P., L. J. Jenkins, Jr., R. A. Jones, R. A. Coon, and J. Siegel. 1970. Repeated and Continuous Exposure of Laboratory Animals to Acrolein. Toxicol. Appl. Pharmacol. 17:726-732.

36. Masri, M. S., A. P. Hendrickson, A. J. Cox, Jr., and F. DeEds. 1964. Subacute Toxicity of Two Chrysanthemumic Acid Esters: Barthrin and Dimethrin. Toxicol. Appl. Pharmacol. 6:716-725.

37. Nakaue, H. S., F. N. Dost, and D. R. Buhler. 1973. Studies on the Toxicity of Hexachlorophene in the Rat. Toxicol. Appl. Pharmacol. 24:239-249.

38. Phillips, W.E.J., J.H.L. Mills, S. M. Charbonneau, L. Tryphonas, G. V. Hatina, Z. Zawidzka, F. R. Bryce, and I. C. Munro. 1978. Subacute Toxicity of Pyridoxine Hydrochloride in the Beagle Dog. Toxicol. Appl. Pharmacol. 44:323-333.

39. Thompson, G. R., R. W. Fleischmann, H. Rosenkrantz, and M. C. Braude. 1974. Oral and Intravenous Toxicity of Δ^9-Tetrahydrocannabinol in Rhesus Monkeys. Toxicol. Appl. Pharmacol. 27:648-665.

40. Verschuuren, H. G., R. Kroes, and E. M. Den Tonkelaar. 1975. Short-Term Oral and Dermal Toxicity of MCPA and MCPP. Toxicology 3:349-359.

5. CHRONIC TOXICITY AND CARCINOGENICITY TESTING

5.1 INTRODUCTION

Tests designed to evaluate chronic toxicity and those designed to evaluate carcinogenic effects have many similar considerations. Both are subject to various design factors, including species, strain, sex, age, and number of the test and control animals; duration of test; route of exposures; dosage levels; and use of data evaluations. Both studies are costly and involve extensive manpower and time requirements. However, the two tests are also different in many ways. There is little uniformity in the literature regarding the purposes, methods, and nomenclature of tests for chronic toxicity. Some investigators seek only the demonstration of a "safe" or "harmless" dose, while other workers attempt to observe the nature and severity of toxic effects as well as to determine a maximum "no-observed-adverse-effect" dose level (Friedman, 1973). In contrast, for carcinogenicity tests, tumor[1] formation and increased tumor incidence are the major end points. Tumor incidence, tumor latency, and in some cases tumor multiplicity are the parameters used to determine sensitivity of the test animals to the carcinogenic challenge. Short-term in vivo tests can also be useful, and several types are included as examples of intermediates between the lifetime studies and the various short-term in vitro tests. Therefore, in the following subsections, chronic toxicity and carcinogenicity tests and the factors that affect them will be discussed together when possible, pointing out both the similarities and dissimilarities. There will also be discussions of topics that pertain only to each individual test. Additionally, some topics usually considered part of chronic toxicity testing, such as reproductive and behavioral effects, neurotoxicity, and teratogenicity, are discussed elsewhere in this report or later publications and are not included in this section.

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[1] In this report, the general term "tumor" will apply to either benign or malignant neoplasms.

5.2 TEST ANIMALS

Various aspects of the selection of species and strain of experimental animal will be discussed in the context of long-term toxicity tests, which include assays for chronic toxicity and carcinogenicity. Species- and strain-specific responses and spontaneous tumor incidence in test animals will be examined through a review of the literature.

5.2.1 Species

Because the usual purpose of chronic toxicity and carcinogenicity testing is to predict adverse effects of chemicals in man, test animals should be chosen that closely resemble man with respect to absorption, distribution, metabolism, excretion, and target site effect of the toxic substance (Weil, 1972). Unless information to the contrary is available from previous metabolic, pharmacodynamic, or subchronic studies, the most sensitive species and strain should be selected for chronic toxicity and carcinogenicity testing (National Academy of Sciences, 1977).

No test animal has been found to be an ideal surrogate for man under all test conditions (Krasovskii, 1976; Rall, 1969; Shubik, 1972); furthermore, great variability of response exists among different species (Hodge et al., 1967). Nevertheless, a variety of species can respond to individual toxicants in a manner useful for chronic testing. Thus, dogs, cats, and nonhuman primates, but not mice or rats, have been found suitable for testing methylmercury compounds (National Academy of Sciences, 1977), and the rhesus monkey is more similar to humans in the dermal absorption of certain compounds such as benzoic acid, hydrocortisone, and testosterone than is the pig, rat, or rabbit (Fancher, 1978). The chicken more closely resembles man than other species tested for response to demyelinating effects of triorthocresyl phosphate and other organophosphorus compounds (Barnes and Denz, 1954). In a similar vein, monkeys are recommended for inhalation experiments because of anatomical similarities to man, cats for compounds likely to produce methemoglobinemia, pigtail monkeys for methanol toxicity, and dogs or rats for cholinesterase inhibition studies (Fancher, 1978).

5.2.1.1 Chronic Toxicity Testing – Despite the need for manlike metabolic responses in test animals, in the ultimate analysis practical considerations drastically limit the choice of animals for chronic testing. Because the test period must encompass a major portion of the life span of the test species, short-lived mammalian species usually receive first consideration. Precedent, convenience, and economic considerations dictate the use of rats or mice in the majority of all chronic toxicity tests (Stevenson, 1979). For example, in more than 100 computer-selected publications on noncarcinogenic chronic toxicity examined during the preparation of this chapter (listed in the Bibliography), the frequency of occurrence of test animals was: rat, 54%; dog, 24%; monkey, 9%; mouse, 6%; rabbit, 4%; guinea pig, 3%; and gerbil, 1%. Only a few of these studies exceeded 2 years in duration. On the basis of animals exposed to the test material over a major portion of their life spans, the above data on animal frequency show a strong bias with respect to short-lived rodents: rat, 88%; mouse, 8%; dog, 2%; and gerbil, 2%.

The dominating effect of practical considerations in the choice of test animals for chronic toxicity testing is further reflected in animals used in 134 long-term studies published in *Toxicology and Applied Pharmacology* between 1959 and 1966 (Benitz, 1970): rat, 43.3%; dog, 38.1%; monkey, 6.7%; mouse, 3.7%; rabbit, 3.0%; chicken and guinea pig, 1.5%; and gerbil, 0.7%. A survey of all studies published in the same journal during 1975 revealed a continuing preference for use of short-lived rodents: rat, 43.7%; mouse, 15.9%; rabbit, 7.6%; dog, 7.5%; primate, 7.3%; guinea pig, 3.7%; human, 3.4%; fish, 3.0%; cat, 2.2%; hamster, 1.1%; chicken, 0.9%; swine, 0.7%; duck, 0.6%; quail, 0.6%; cow, 0.4%; sheep, 0.4%; frog, 0.2%; goat, 0.2%; sea lion, 0.2%; and snail, 0.2% (Fancher, 1978).

When relatively long-lived nonrodent animals are the test species of choice and it is not feasible to expose the animals to the test material over a major fraction of their life span, some authorities recommend conducting kinetic studies to determine when steady-state tissue concentrations of the test chemical and its metabolites have been achieved. Treatment of the animal for a substantial period after attaining steady-state kinetics would then partially substitute for a lifetime study and provide added assurance of the validity of experimental conclusions (National Academy of Sciences, 1977; World Health Organization, 1978).

When a rodent is chosen as the principal test animal in a chronic toxicity test, most authorities recommend including a second species that is not a rodent in order to reveal a broader range of toxic effects (Barnes and Denz, 1954; Zbinden, 1973). Usually, the dog is chosen (*Federal Register*, 1978; Goldenthal, 1968; Page, 1977*b*), but other species may be selected if their metabolic processes are thought to resemble those of man.

5.2.1.2 Carcinogenicity Testing — Rodents, mainly the rat, mouse, and Syrian hamster, are generally selected for large-scale screening of suspected carcinogens (Ministry of Health and Welfare Canada, 1975; National Academy of Sciences, 1975; Page, 1977*b*). Utilization of these species is based neither on the established similarities to man nor on biochemical, physiological, or anatomical characteristics but on the ability to test large numbers of compounds in these animals in a relatively short time. Although the susceptibility of simians and the dog to chemical carcinogenesis has been established with several groups of chemicals, the long latency period in these species discourages their use in carcinogenicity screening (Ministry of Health and Welfare Canada, 1975; National Academy of Sciences, 1975; U.S. Food and Drug Administration, 1971). Dogs and monkeys may be useful for extrapolation purposes, but only in highly select situations (Page, 1977*b*).

The selection of the most appropriate animal system is an important factor in tests for carcinogenicity, and species differences in response to various chemical carcinogens must be a major consideration in that selection. Responses of various mammalian species to chemical carcinogens within selected chemical classes are reviewed in the following subsections.

5.2.1.2.1 Polynuclear aromatic carcinogens — Tumor induction with polynuclear aromatic compounds has been demonstrated in a variety of species using different routes of administration. The following section includes comparisons of species' responses to a few of these compounds. The tumorigenic response of animals to a particular carcinogen can vary according to route of administration of the test material, and this is demonstrated in Sect. 5.3; thus, valid comparisons can be made between species only when the same route of exposure is used. Therefore, the studies are grouped according to mode of administration of the carcinogen.

Tumors have been induced in many species by subcutaneous injection of polycyclic aromatic carcinogens or by subcutaneous implantation of special discs which have been impregnated with the carcinogens. For instance, when injected subcutaneously into newborn mice, 7,12-dimethylbenz(*a*)anthracene has been found to be a potent leukemogen (Pietra, Rappaport, and Shubik, 1961). Of 27 Swiss albino mice injected with 30 to 40 μg dimethylbenzanthracene in 1% gelatin, 29.6% developed malignant lymphoma by 12 to 27 weeks after injection, and 85.4% developed pulmonary tumors. However, when newborn Lewis rats were injected subcutaneously with 10 to 1000 μg of dimethylbenzanthracene, the only tumors related to the treatment were sarcomas induced at the site of injection (Toth and Shubik, 1963). The number and latent periods of the tumors were dose related. Thus, it seems that newborn Swiss mice respond systemically to subcutaneously administered dimethylbenzanthracene, while newborn Lewis rats respond locally.

Pott, Brockhaus, and Huth (1973) found the subcutaneous tissue of the NMRI mouse to be more sensitive than that of the Wistar rat to benzo(*a*)pyrene. Female NMRI mice developed malignant sarcomas at the site of injection of benzo(*a*)pyrene in tricaprylin (Pott, Tomingas, and Misfeld, 1977). Single doses of 3.3 to 270 μg of the carcinogen were administered. A linear dose relationship was observed in the range of 3.3 to 90 μg for an induction time of 20 to 41 weeks. Survival time was 21 to 84 weeks, and the tumor incidence was 8% to 80%.

When sensitive strains of Syrian hamsters were injected subcutaneously with 500 μg of benzo(*a*)pyrene in tricaprylin, sarcomas were palpable at 20 weeks (Homburger et al., 1972). In the experiments of Pott et al. and Homburger et al. the average induction time for the appearance of subcutaneous tumors was the same for mice and hamsters. However, the dose required to produce tumors in hamsters in 20 weeks was two times the dose required to produce tumors in mice in the same time. If one assumes that the dose response in hamsters is linear, the mice would be more sensitive. In the same hamster study, 500 μg of 3-methylcholanthrene induced sarcomas at the injection site in 17 weeks; in an earlier experiment (Homburger and Hsuch, 1970), sarcomas appeared in hamsters only 3 weeks after subcutaneous injection of 7,12-dimethylbenzanthracene in tricaprylin.

Hartley guinea pigs, on the other hand, exhibited relatively prolonged induction times of 7 to 17 months after methylcholanthrene was injected into the abdominal wall (Dale et al., 1973). One or two doses of 4 mg of the carcinogen in sesame oil produced sarcomas in 30% of the animals surviving for 6 months. The dose of methylcholanthrene administered was 8 to 16 times higher than the dose that, in the study of Homburger et al. (1972), induced subcutaneous tumors in hamsters in 17 weeks. Thus, the guinea pig seems relatively resistant to subcutaneous tumor induction with 3-methylcholanthrene.

A few years earlier O'Gara and Kelly (1965) attempted to produce tumors in rhesus and cynomalgus monkeys using conditions known to produce malignant tumors in rodents. Dibenzo(*a*,*i*)pyrene and 3-methylcholanthrene injected subcutaneously or intradermally induced local keratoacanthomas, papillomas, and giant cell granulomas but no sarcomas or carcinomas.

The squirrel monkey (*Saimiri sciresus*) is the second most widely used subhuman primate in biomedical research, and by 1969 no reports had been made of tumor induction in this animal. Steinmuller, Dillingham, and Prehn (1969) attempted to induce tumors with subcutaneously implanted discs of paraffin impregnated with 5% 3-methylcholanthrene (one disc per 25 g of body weight). No tumors or neoplastic changes were found 4 years later in any of eight monkeys tested. In addition, autochthonous skin fragments implanted subcutaneously with 3-methylcholanthrene crystals failed to give rise to tumors in 4 years in the three monkeys treated. No signs of toxicity were seen in monolayers of monkey kidney epithelial cells grown in vitro with 5% 3-methylcholanthrene discs during 3 weeks of exposure, although Alfred et al. (1964), using identical techniques, had produced marked toxicity in mouse cells.

The most frequent explanation for the differences in tumor response between rodents and primates is based on the assumption of a direct relationship between the induction time of tumors and the life span of the animal. However, experiments of Sugiura, Smith, and Sunderland (1956) do not support this but tend to emphasize differences in sensitivity among the species to a particular carcinogen. Thus, skin painting with MH101, a high boiling catalytically cracked oil, in repeated doses produced papillomas in six rhesus monkeys and, later, malignant change in three of them. This was the first demonstration of tumor induction in *Macacus rhesus*. In the same experiment, mice and rabbits developed papillomas and cancers, but rats and guinea pigs did not. Table 5.1 demonstrates the lack of a relationship between life span and tumor induction in various species (e.g., papilloma induction required 1/25 of the life span of the mouse, 1/18 of the life span of the monkey, but only 1/70 of the life span of the rabbit). Steinmuller, Dillingham, and Prehn (1969) concluded from the experiment of Sugiura, Smith, and Sunderland (1956) and from their own data that the species-specific response to carcinogens was more evident than a relationship between induction time and life span. They also suggested that primates are more resistant than rodents to the polycyclic hydrocarbons.

In contrast to squirrel monkeys and rhesus monkeys, primates lower in the phylogenetic scale seem to be responsive to the carcinogenic effects of polycyclic hydrocarbons (Adamson, Cooper, and O'Gara, 1970) with the difference in response occurring approximately at the evolutionary level of the marmoset. Levy (1963) injected a marmoset subcutaneously with 2 mg of 3-methylcholanthrene, and a fibrosarcoma appeared in 10 months; Noyes (1969) induced a fibrosarcoma and a rhabdomyosarcoma in 16 months after injection of benzo(*a*)pyrene and dimethylbenzanthracene, respectively, into opposite flanks of a cottontop marmoset. Also, Noyes (1968) observed fibrosarcomas in three tree shrews (*Tupia glis*) 6 months after injecting benzo(*a*)pyrene. In a similar study, Adamson, Cooper, and O'Gara (1970) injected six tree shrews with single subcutaneous doses of 10 mg methylcholanthrene dissolved in 1 mL olive oil. At the same time, 12 galagos were injected intradermally or subcutaneously with 3 to 10 mg benzo(*a*)pyrene. The three tree shrews that survived developed tumors at the injection site, the first appearing 14 months after injection. Of the surviving 12 galagos, one developed a tumor at the site of injection in 26 months. The fibrosarcomatous nature of the tumors

Table 5.1. Some factors in the experiments with MP 101 upon a variety of species

Species	Time to first appearance (days)		Dose (g)	Approximate weight of animal (g)	Dose per kg (g)	Area treated[a] (cm^2)	Dose per cm^2 (g)	Approximate life span (years)
	Papilloma	Cancer						
Mouse	20 (1/25)[b]	70 (1/10)	0.015	25	0.6	1	0.015	2
Rat	0	0	0.1	200	0.5	4	0.025	3
Guinea pig	0	0	0.1	400	0.25	4	0.025	6
Rabbit	26 (1/70)	411 (1/4)	0.5	3000	0.17	16	0.031	5
Monkey	322 (1/18)	1373 (1/4)	0.2-1.0	7000	0.03-0.14	4-35	0.05-0.03	16

[a] Refers to a single painted site.

[b] Numbers in parenthesis represent portion of life span required for tumor induction.

Source: Adapted from Suguira, Smith, and Sunderland, 1956. Reprinted with permission of the publisher.

induced in these species [and the failure to induce sarcomas in rhesus and cynamolgus monkeys (O'Gara and Kelly, 1965) and in squirrel monkeys (Steinmuller, Dillingham, and Prehn, 1969)] led Adamson et al. to suggest that prosimians, which include tree shrews, lemurs, lorises, and tarsiers, may resemble rodents more than the higher primates in their reactions to polycyclic hydrocarbon carcinogens.

Although many skin carcinogenesis experiments have been conducted using the two-stage, initiation-promotion technique, promotion studies are omitted from this section to simplify discussion. Skin tumor induction times are compared for mice, rats, and hamsters in the following examples.

Weekly cutaneous applications of 0.1% (or 0.025 mg per dose) 9,10-dimethyl-1,2-benzanthracene to the skin of noninbred mice induced papillomas in 5 to 6 weeks and carcinomas in 20 to 40 weeks (Neiman, 1968). The induction times for both papillomas and carcinomas were reduced considerably by presensitization with nontumorigenic doses of the carcinogen. In a later study with C57 Bl/6J mice, Wislocki et al. (1977) noted the appearance of squamous cell carcinomas after 30 weeks of biweekly applications (15 skin paintings) of benzo(*a*)pyrene.

Similar experiments have been conducted in rats and hamsters. Multiple weekly doses of 1% dimethylbenzanthracene applied to the skin of hamsters gave rise to papillomas and squamous cell carcinomas. The average latent period was 16 weeks (Della Porta et al., 1956). Doses of 0.5% of the same carcinogen applied to the skin of Fischer rats produced squamous cell carcinomas after 6 months of skin painting (Zackheim, 1964). Table 5.2 summarizes the results of the three experiments with dimethylbenzanthracene. The differences in dose administered in the three studies increase the difficulty of comparing responses of the animals. Nonetheless, the limited data indicate that mice are more sensitive than hamsters and rats to skin carcinogenesis with 9,10-dimethyl-1,2-benzanthracene, supporting the view that mouse skin generally exhibits greater sensitivity than rat skin to carcinogenesis with polycyclic aromatic hydrocarbons (Weisburger, 1976).

Tumors of the respiratory tract have been induced by polycyclic aromatic hydrocarbons in the various species by special techniques,

Table 5.2. Induction of skin carcinomas in mice, hamsters, and rats with weekly applications of 9,10-dimethyl-1,2-benzanthracene

Species	Dose	Induction time	Number of applications
Inbred mice	0.1% (0.25 mg)	5 to 10 months	20 to 40
Syrian hamsters	1%	4 months, average	16
Fischer rats	0.5%	6 months, average	24

which include intratracheal injection. In the first report of tumor induction by the intratracheal route of administration, weekly instillations of 50 μg dimethylbenzanthracene in 1% gelatin for up to 45 weeks produced tracheobronchial carcinomas in hamsters (Della Porta, Kolb, and Shubik, 1958). In a recent study Kektar et al. (1978) instilled benzo(*a*)pyrene, weekly, in Syrian golden hamsters at doses of 0.1, 0.33, or 1.0 mg (in bovine serum albumin). Respiratory tract neoplasms were found in 19% to 22% of the males and 20% to 40% of the females treated. Survival times of 10 to 40 weeks were dose related, but tumor incidence was not.

Although hamsters have been the animal of choice for intratracheal studies in the past, pneumonia-free specific pathogen-free (SPF) rats are now used frequently because of their low incidence of infectious diseases (Nettesheim and Griesemer, 1978). Davis et al. (1975) induced squamous carcinomata in the lungs of Wistar rats with intratracheal injections of benzo(*a*)pyrene. Using infusine as the vehicle, 0.5-, 1.0-, and 2.0-mg doses were administered once every 2 weeks. Twenty-four of 48 rats developed squamous neoplasms of the lung. Unlike the results with the hamster system (Ketkar et al., 1978), the tumor incidence in rats was dose related. Tumors have also been induced by intratracheal instillation of carcinogens in mice (Nettesheim and Hammons, 1971), in prosimian galagos (Crocker et al., 1970), and in rabbits (Hirao et al., 1968).

Another mode of administration of chemical carcinogens is by direct injection into the salivary gland. Glucksman and Cherry (1971) injected 0.1 mL of a 2% suspension of 9,10-dimethyl-1,2-benzanthracene in acetone into the salivary glands of male and female Lister rats (18 males and 10 females) and observed carcinomas and/or sarcomas of the glands in 16 of 18 males and 10 out of 10 females. Carcinomas peaked at 100 days, and sarcomas appeared as late as 770 days.

Wigley, Amos, and Brookes (1978) induced tumors in the salivary glands of C57BL male mice by direct injection of 0.1, 0.5, or 2.0 mg benzo(*a*)pyrene. Forty percent of the animals injected with 0.1 mg, 90% injected with 0.5 mg, and 56% injected with 2 mg developed fibrosarcomas, carcinomas, and/or rhabdomyosarcomas. (Two lymphosarcomas developed at sites other than the injection site.) The first two tumors appeared approximately 110 days after injection of benzo(*a*)pyrene. The responses of mice and rats to polynuclear aromatic carcinogens injected locally into the salivary glands seem to be similar.

A broad spectrum of tumor types can be elicited in laboratory animals with polycyclic aromatic hydrocarbons administered systemically. For example, mice have been shown to develop gastric carcinomas, pulmonary adenomas, and/or leukemias and lymphomas following oral administration of benzo(*a*)pyrene; the organ specificity of the response is clearly related to strain (Rigdon and Neal 1966; Rigdon et al., 1969). To be specific, of 81 male Wistar rats given daily intragastric instillations of 5 mg 3-methylcholanthrene, 28% developed 26 malignant neoplasms (10 mammary carcinomas, 4 sebaceous gland carcinomas, 7 cutaneous carcinomas, and 5 leukemias). Of 122 female rats, 115 (98%) developed 117 malignant neoplasms (106 mammary carcinomas, 10 leukemias, 1 sebaceous gland carcinoma, and no skin carcinomas) (Gruenstein, Meranze, and Shimkin, 1966).

Huggins, Grand, and Brillantes (1961) observed preferential mammary cancers in 100% of the 50-day-old Sprague-Dawley female rats 28 to 59 and 34 to 70 days after feeding them sesame oil solutions of 20 and 10 mg of 7,12-dimethyl-benzanthracene, respectively. Only ten animals per group were used. Control groups were not included in the experiment, but the investigators reported that only two mammary cancers had been observed in 20 000 females less than 8 months old in their colony.

Thus, female rats seem to be highly susceptible to mammary carcinogenesis following oral administration of polycyclic aromatics.

The carcinogenic response of hamsters to 3-methylcholanthrene was tested by Homburger et al. (1972), who administered, by gavage, 5 mg of the chemical three times per week for 17 weeks and observed carcinomas in males of the most susceptible strain tested 16 to 31 weeks later. Tumors were found in the forestomach (20%), stomach (40%), small intestine (45%), and large intestine (65%). Eighty-seven percent of the female hamsters of that strain developed mammary tumors of various histological types.

5.2.1.2.2 Aromatic amines — Bladder cancer in humans has been reported in all countries with established chemical industries. A correlation between aromatic amine exposure and human bladder cancer was first reported by Rehn in 1895. Tumor induction in animals by aromatic amines and subsequent epidemiological studies have confirmed the correlation reported by Rehn. Experimental details for the studies discussed in this section are shown in Table 5.3.

Early attempts to produce bladder cancer in various species were unsuccessful, but in 1937 Heuper and Wolfe reported bladder tumors in dogs following oral and subcutaneous administration of 70 g of 2-naphthylamine. The tumors were first diagnosed by use of cystoscopy 21 months after the start of treatment.

Nelson and Woodard (1953) demonstrated the susceptibility of dogs to *o*-aminoazotoluene (*o*-AAT). Administered orally for 30 to 62 months, *o*-AAT produced urinary bladder tumors in two of four animals studied. The other two dogs developed liver and gallbladder tumors.

Another aromatic amine, 4-aminodiphenyl, was used by Walpole, Williams, and Roberts (1954) to produce bladder cancer in the dog. Cumulative doses of 30 to 33 g, orally administered, induced tumors in the two dogs tested within 2½ years. Thirty control beagles developed no bladder tumors during 3 to 9 years of observation.

Walpole's results were confirmed in a 3-year study by Deichmann et al. (1965), who were able to induce bladder tumors in 100% of six dogs with smaller cumulative doses (5.4 to 7.3 g per dog) of 4-aminodiphenyl.

Table 5.3. Tumor induction in various species with aromatic amines

Chemical/species	Dose	Duration of exposure (weeks)	Route of exposure	Tumor incidence	Tumor latency (weeks)	Reference
2-Naphthylamine						
Dog	70 g		Oral; subcutaneous	Bladder	84	Heuper and Wolf, 1937
Syrian hamster	1.0%	~36	Oral, diet	30% Bladder carcinomas	40	Saffiotti, 1966
Mouse	1-2 mg per pellet	22-39	Bladder pellet	0/8		Bonser et al., 1952
IF mouse	200 mg/kg body weight in arachis oil twice weekly	30-72	Oral, gavage	10/25 Liver cholangiomas	50-72	Bonser et al., 1952
CBA mouse	120 mg/kg body weight in arachis oil twice weekly	30-89	Oral, gavage	13/23 Hepatomas; 1 cholangioma	50-89	Bonser et al., 1952
CBA mouse	160 mg/kg body weight once weekly	40-89	Oral, gavage	11/26 Hepatomas	60-89	Bonser et al., 1952
IF mouse	Saturated solution in benzene	40-99	Skin	0/25		Bonser et al., 1952
Albino rat	310 mg/kg body weight per week	20-90	Oral, diet (reduced protein)	3/15 Bladder papillomas	60-90	Bonser et al., 1952
Rabbit	200 mg twice per week	140-272	Oral, spoon	1/6 Bladder papilloma	247	Bonser et al., 1952
2-Acetylamino-fluorene						
Dog	4-12 mg/kg body weight per day	272-364	Oral, diet	4/4 Bladder, liver	272-364	Morris and Eyestone, 1953
Mouse	0.05%	56	Oral, diet	14/49 Bladder carcinoma or papilloma	48	Miller, Miller, and Enomota, 1964
Guinea pig	0.045% or 0.032%	112-128	Oral, diet	0/16		Miller, Miller, and Enomota, 1964
	1.5 and 1.6 mg per 100 g body weight 2-3 times per week	9-22	Intraperitoneal	0/26		Miller, Miller, and Enomota, 1964
Monkey	Dosage increased with time from 10-200 mg/kg body weight per day, 5 days per week	52-182	Oral, diet	0/16		Dyer, Kelly, and O'Gara, 1966

Table 5.3. (continued)

Chemical/species	Dose	Duration of exposure (weeks)	Route of exposure	Tumor incidence	Tumor latency (weeks)	Reference
2-Acetylaminofluorene						
Hamster	0.03%	30	Oral, diet	3/18 Liver bile duct carcinomas	52	Miller, Miller, and Enomota, 1964
	1.5 mg per 100 g body weight per day 3 times per week	24-32	Intraperitoneal	1/18 Small intestine adenocarcinoma	64	Miller, Miller, and Enomota, 1964
o-Aminoazotoluene						
Hamster	0.1%	49	Oral, diet	20/25 Bladder 19/24 Liver cell 3/15 Mammary	45	Tomatis, Della Porta, and Shubik, 1961
Rat	1.4-3.4 g total dose	21-49	Oral	7/8 Hepatomas	21	Yoshida, 1932
C3H mouse Strain C mouse Strain A mouse F_1 hybrids of above	10 mg once per 4 weeks in glycerol	40	Subcutaneous	Varied with strain Hepatic Pulmonary Hemangio-endotheliomas		Andervont, Grady, and Edwards, 1942
Dog	20 mg/kg per day	8	Oral	0/5 (all died with liver damage)		Nelson and Woodard, 1953
	5 mg/kg per day	128-248	Oral	2/5 Bladder carcinomas		Walpole, Williams, and Roberts, 1954
4-Aminodiphenyl						
Dog	Varied 5-20 mg/kg body weight 6 days per week (Total = 2.9-3.3 g/kg)	140	Oral, capsule	2/2 Bladder		Walpole, Williams, and Roberts, 1954
	1.0 mg/kg body weight 5 times per week	156	Oral, capsule	6/6 Bladder (4 malignant, 2 benign)		Deichmann et al., 1965
Rat	3.6-5.8 g/kg body weight total	36-53	Subcutaneous	17/23 (Variety of sites. No bladder tumor.)		Walpole, Williams, and Roberts, 1952

Similar doses of 2-naphthylamine failed to produce neoplasms in six dogs, thus establishing 4-aminodiphenyl as the more potent carcinogen in this study.

Morris and Eyestone (1953) induced liver and bladder tumors in each of four dogs fed a total dose of 90 to 198 g of 2-acetylaminofluorene, administered orally for 68 to 91 months. Experiments in which bladder cancer has been induced in dogs by other aromatic amines are listed in Table 5.4.

A distinct difference exists among the various species in their responsiveness to carcinogenesis by aromatic amines. The following demonstrate some of those differences: (1) 2-naphthylamine produced bladder cancer in hamsters (Saffiotti et al., 1966) but not in rats, mice, and rabbits (Bonser et al., 1952); (2) *o*-ATT produced bladder cancer in hamsters (Tomatis, Della Porta, and Shubik, 1961), but not in rats (Yoshida, 1932) or mice (Andervont, Grady, and Edwards, 1942; Andervont, 1950); (3) 4-aminodiphenyl failed to induce bladder tumors in rats (Walpole, Williams, and Roberts, 1952); and (4) 2-fluorenylacetamide produced bladder tumors in mice but not in hamsters, guinea pigs (Miller, Miller, and Enomota, 1964), or monkeys (Dyer, Kelly, and O'Gara, 1966).

Because of the relative resistance encountered in other species, it is felt that the dog should be used for testing carcinogenicity of substances related to the aromatic amines (U.S. Food and Drug Administration, 1971). However, the hamster can develop aromatic amine-induced bladder tumors having short latent periods and may be a likely substitute (Gak, Graillot, and Truhaut, 1976).

5.2.1.2.3 *N*-Nitroso compounds — About 100 *N*-nitroso compounds have been shown to be carcinogenic in experimental animals. The carcinogenic properties of some of these compounds are compared in different species.

Diethylnitrosamine and dimethylnitrosamine — In 1967 Schmähl and Osswald reviewed the effects of diethylnitrosamine in 11 different animal species, paying particular attention to the organotropic activity of the carcinogen. The data of Schmähl and Osswald (1967) and those of other investigators are summarized in Table 5.5, which includes the total dose (mg per kg of body weight) required to produce cancer in the various species. Tumors were induced in all species and in almost 100%

Table 5.4. Compounds, not previously listed, which have produced bladder cancer in the dog

Compound	Individual doses	Total dose per animal that produced tumors (g)	Incidence of tumors	Latent period
Benzidine	200 mg per dog per day (in capsules), 6 days/week for 15 months, followed by 300 mg per dog, 6 days/week for 45 months; then treatment discontinued	325	1 of 6 mongrels (1 male, 5 females)	7.5 years
4-Dimethylamino-azobenzene	20 mg/kg per dog per day[a] (dry or in corn oil in capsules)		2 (1 male and 1 female) of 10 mongrels	3 years, 2 months to 4 years
4-Nitrobiphenyl	0.3 g per dog three times a week (in capsules)	98 to 129	3 of 4 female mongrels	2 years, 1 month to 2 years, 9 months
2-Naphthylamine plus 4-nitrobiphenyl	0.1 g of each compound per dog, three times a week (in capsules)	Total: 17 to 20	5 of 5 female beagles	2 years, 5 months to 2 years, 7 months
2-Naphthylamine plus 4-nitrobiphenyl plus 4-aminobiphenyl	0.1 g of each compound per dog, three times a week (in capsules)	Total: 22 to 25	3 of 3 female beagles	2 years, 1 month to 2 years, 4 months

[a] It is not clear whether a certain dosage per day was given five or seven times per week.

Source: Adapted from Deichmann and Radomski, 1963. Data collected from several sources.

Table 5.5. The carcinogenic action of diethylnitrosamine in different animal species

Species	Route of administration	Daily dose (mg/kg)	Total dose (D_{50}) (mg/kg)	Type of tumor
Mouse	Oral	3	871 ± 124	Hemangioendotheliomas of the liver
Rat	Oral	3	700 ± 53	Hepatocarcinomas
Hamster	Oral	40 (weekly)	640	Hepatocarcinomas
Guinea pig	Oral	3	1200 ± 100	Hepatocarcinomas
Rabbit	Oral	3.4	2500	Hepatocarcinomas
Dog	Oral and subcutaneous	3	560	Leimyosarcoma of the liver
Pig	Oral	4.4	1400	Reticulosarcomas of the liver
Monkey	Oral	2 to 50	1400 to 25 700	Hepatocarcinomas
Grass parakeet	Intramuscular	70 (weekly)	2800 ± 400	Hepatocarcinomas
Brachydanio rerio	Oral	10 to 100 ppm	10 to 13 weeks	Hepatocarcinomas and cholangiomas
Trout				Hepatocarcinomas

Source: Adapted from Schmähl and Osswald, 1967. Reprinted with permission of the publisher.

of the animals tested. Rabbits, dogs, and pigs treated with approximately 3 mg/kg diethylnitrosamine developed cirrhosis and liver cancer, while mice, rats, and guinea pigs developed only liver cancer.

Although Schmähl and Osswald were unable to produce hepatomas in monkeys, they referred to Kelly et al. (1966), who had done so in 6 of 15 monkeys (cynamolgus, rhesus, and capuchin) with oral doses of diethylnitrosamine administered for more than 1 year. Experimental details of this and the following studies with diethylnitrosamine and dimethylnitrosamine are listed in Table 5.6.

Two cercopithecus monkeys given 1.6 and 2.2 g of diethylnitrosamine intraperitoneally also developed hepatocarcinomas, with induction times of 25 and 27 months (Kelly et al., 1966).

The monkeys in the preceding experiments did not develop tumors in organs other than the liver. A strain of inbred guinea pigs, however, developed tumors in both the lung and the liver when diethylnitrosamine was administered in their drinking water (Argus and Hoch-Ligeti, 1963). In these animals the first liver carcinoma and the first lung papilloma were diagnosed at 16 weeks and 45 weeks, respectively. In a later study with outbred Hartley guinea pigs, diethylnitrosamine in the water induced only hepatomas, with a mean induction time of 15 months (Dale et al., 1973).

Rats, on the other hand, developed tumors in a variety of organs following oral administration of diethylnitrosamine (Lijinsky and Taylor, 1978). Diethylnitrosamine was administered in drinking water to groups of 6 to 15 Sprague-Dawley rats for up to 29 weeks. The animals survived for 33 weeks, and 100% of them developed tumors of the liver (carcinomas and sarcomas), nasal turbinates, and/or esophagus. In a study by Reuber (1976), even more extensive lesions were observed in Buffalo strain rats of various ages fed diethylnitrosamine in their diet for 26 weeks. Malignant tumors appeared in the esophagus, liver (carcinomas and sarcomas), and prostate gland; the type and distribution of tumors depended on the sex and age of the animals. The carcinogen was incorporated into the diet in the amount of 0.0114%, and the first tumors were seen 24 to 36 weeks after the start of the experiment.

Similar to rats, BALB/c mice developed tumors of the liver and esophagus as a result of ingesting diethylnitrosamine in drinking water

Table 5.6. Tumor induction in various species with diethylnitrosamine and dimethylnitrosamine

Chemical/species	Dose	Duration of exposure (weeks)	Route of exposure	Tumor incidence	Tumor latency (weeks)	Reference
Diethylnitrosamine						
Monkey	2-5 mg/kg body weight/day Total: 6-24 g	108	Oral	6/18 hepatomas	92	Kelly et al., 1966
Monkey	20-40 mg per 2 weeks Total: 1.6-2 g	108	Intraperitoneal	2/2 hepatomas	104	Kelly et al., 1966
Inbred guinea pig	1.1-4.2 mg/day Total: 200-300 mg	16 to 40	Oral	14/15 hepatomas 7/15 lung tumors 2/15 bronchial papillomas	16 to 46	Argus and Hoch-Ligeti, 1963
Outbred Hartley guinea pig	About 3 mg/day 3 days/week	Up to 68	Oral	35/35 hepatomas	60	Dale et al., 1973
Sprague-Dawley rat	Total: 203 mg	29	Oral	10/12 liver tumors 2/12 nasal turbinate tumors 6/12 esophageal papilloma 1/12 esophageal carcinoma	About 20	Lijinsky and Taylor, 1978
Buffalo strain rat (12 weeks old)	0.0114% in diet	26	Oral	1/7 prostate tumor 12/26 esophageal carcinoma Unclear liver	24 to 36	Reuber, 1976
Mice BALB/c	Total: 515 mg/kg body weight	41	Oral	4/15 liver hemangiosarcomas 6/15 esophageal tumors 3/15 stomach tumors 0/15 lung tumors	Unclear	Clapp, Tyndall, and Otten, 1971
	Total: 1010 mg/kg body weight	41	Oral	14/60 liver hemangiosarcomas 35/60 stomach tumors 2/60 lung tumors	Unclear	Clapp, Tyndall, and Otten, 1971

Table 5.6 (continued)

Chemical/species	Dose	Duration of exposure (weeks)	Route of exposure	Tumor incidence	Tumor latency (weeks)	Reference
Mice, Swiss	6 mg/kg body weight/week	10	Intraperitoneal	30/38 lung tumors 4/38 vascular tumors 3/38 forestomach papillomas 0/38 kidney tumors 10/38 lymphomata 1/38 mammary adenocarcinoma	66	Cardesa et al., 1974
Dimethylnitrosamine						
Mice, BALB/c	Total: 300 mg/kg body weight	Up to 41	Oral	3/15 hemangio-sarcoma 7/15 lung tumors	Unclear	Clapp, Tyndall, and Otten, 1971
Mice, Swiss	6 mg/kg body weight/week	10	Intraperitoneal	37/39 lung tumors 12/39 vascular tumors 0/39 forestomach papilloma 4/39 kidney tumors 4/39 lymphomata 1/39 mammary adenocarcinoma	59	Cardesa et al., 1974
Chinese hamster	0.89-3.54 mg/kg body weight/week Total: 31-105 mg		Subcutaneous	100/108 liver tumors 1/108 lung tumors 1/108 nasal tumor	26	Reznik, 1975

(Clapp, Tyndall, and Otten, 1971). Tumors were also found in the lung and stomach. Dimethylnitrosamine, however, produced tumors only in the lung and liver. Differences in susceptibility of the lung to diethylnitrosamine (3% tumor incidence) and dimethylnitrosamine (47% tumor incidence) indicate that different enzyme systems may be activated for metabolism of each of the chemicals and/or that the system for metabolizing diethylnitrosamine is not present in sufficient quantity in the lung of the BALB/c mouse. Swiss mice, a strain having a 26% spontaneous lung tumor incidence, also developed more lung tumors after intraperitoneal injection of dimethylnitrosamine than after diethylnitrosamine, but the difference in tumor incidence was less pronounced (Cardesa et al., 1974). Liver tumors in both strains of mice were of vascular rather than of hepatocellular origin. A similar response was reported in Chinese hamsters injected subcutaneously with three doses of dimethylnitrosamine (Reznik, 1975). Tumors were mainly in the liver and were all of vascular origin (hemangioendotheliomas). Tumor incidence was nearly 100% and was not dose dependent; the first liver tumor occurred in the 26th experimental week.

Thus, in the above examples the most obvious species-related difference in the response of animals to diethylnitrosamine and dimethylnitrosamine was in the type of liver tumors induced. Those in monkeys, rats, guinea pigs, and hamsters were of vascular and/or liver cell origin, while those in mice were of vascular origin only.

Other *N*-nitroso compounds — In separate studies, species differences were observed between rats and hamsters treated with diisopropanolnitrosamine (Mohr, Reznik, and Pour, 1977; Pour, Kruger, and Althoff, 1974, 1975). Sprague-Dawley rats were given subcutaneous injections of 178 to 1425 mg per kg of body weight once weekly for 20 weeks, and hamsters received 125 to 500 mg per kg of body weight per week for life. The chief differences noted were: (1) pancreatic tumors were diagnosed in all hamsters, but only one such tumor was observed among 150 rats; (2) cholangiomas or cholangiocarcinomas were found in the hamster but not in the rat; and (3) adenomas and adenocarcinomas were found in the thyroid glands of rats but not in hamsters.

Lijinsky et al. (1967, 1970) tested the carcinogenicity of a cyclic nitrosamine, nitroazetidine, in rats, mice, and hamsters. Five milligrams of the compound per day administered in drinking water for 46 days (total dose 230 mg) induced tumors in 15 out of 20 Wistar rats, and 0.5 mg per day (total dose 23 mg) induced tumors in 15 out of 40 mice. Hamsters were resistant to total doses of up to 500 mg. However, both rats and hamsters were responsive to tumor induction by another cyclic nitrosamine, nitrosoheptamethyleneimine (Lijinsky et al., 1967, 1970).

It may be concluded from the above examples of *N*-nitroso compound carcinogenesis that the organ specificity of these chemicals is approximately the same in all species that respond to them, regardless of route. However, differences do exist in general susceptibility of the various species to specific compounds of this class of chemicals.

5.2.1.2.4 Miscellaneous compounds — Brief mention is made below of species differences encountered in carcinogenesis studies with miscellaneous compounds of chemical classes other than those previously mentioned.

Minerals and Metals — Exposure in inhalation chambers to amosite, crocidolite, and chrysotile was the basis for a comparison of the various responses of rats (Charles River), guinea pigs (Camm-Hartley), rabbits (Shankin Farms Dutch), mice (Swiss), and gerbils (Mongolian) of both sexes (Reeves, Puro, and Smith, 1974). The animals were exposed 4 h per day, 4 days per week for 2 years (1480 h cumulative total exposure). Some animals were sacrificed after 3 to 6 months, but most were allowed to survive until the exposure period ended. Table 5.7 contains results of the study.

Reeves and associates were the first to obtain lung carcinoma and pleural mesothelioma following exposure by inhalation to amosite. Rats seemed to be the only species that displayed an increased incidence of neoplasia of the respiratory tract (mice were excluded because of their tendency to develop spontaneous bronchial cancers). It was also noted that after equivalent asbestos exposure, pulmonary ferruginous bodies were abundant in the guinea pig and rare in the rat. The authors suggested the frequency of ferruginous bodies is inversely related to carcinogenic effects attributable to asbestos inhalation.

Table 5.7. Carcinogenicity of different forms of asbestos in various species

Species	Chrysotile		Crocidolite		Amosite	
	Number surviving	Effect	Number surviving	Effect	Number surviving	Effect
Mouse[a]	19	None	18	Two papillary carcinomas of bronchus	17	None
Gerbil	50	None	49	None	51	None
Rat	43	One papillary carcinoma of lung One squamous carcinoma of lung One mesothelial fibrosarcoma of mediastinum	46	One adenocarcinoma of lung Three squamous carcinomas of lung One papillary adenocarcinoma of lung	46	One metastatic osteosarcoma of lung One mesothelial fibrosarcoma of lung and pleura One fibrous mesothelial of pleura
Rabbit	11	None	9	None	9	None
Guinea pig	14	None	14	None	13	None

[a] Control mouse had one papillary carcinoma of the bronchus. All other controls were free of neoplasma.

Source: Adapted from Reeves, Puro, and Smith, 1974. Reprinted with permission of the publisher.

Sunderman (1971) reviewed the carcinogenic effects of beryllium, cadmium, chromium, cobalt, iron, lead, nickel, selenium, zinc, and titanium in various species by different routes of exposure.

1,1-Trichloro-2,2-bis(p-chlorophenyl) ethane (DDT) — DDT has been shown to be primarily a hepatocarcinogen in mice, although tumors have developed in other organs of more susceptible mouse strains (Innes et al., 1969; Terracini et al. 1973). Tomatis and Turusov (1975) demonstrated that the tumorigenicity of DDT in mice is dose related. In rats, however, DDT has been noncarcinogenic (Cameron and Cheng, 1951) or marginally carcinogenic (Deichmann et al., 1967) and has had no effect on hamsters and monkeys (Agthe et al., 1970; Durham, Ortega, and Hays, 1963).

Aflatoxin B_1 — The hepatocarcinogenicity of aflatoxin B_1 had been demonstrated in rats (Newberne and Rogers, 1972; Wogan and Shank, 1971) but not in mice until Vessilnovitch et al. (1972) induced heptatomas in newborn mice with intraperitoneal injection of a total of 1.5 to 6 µg per g of body weight (in one to five doses). Tumors were observed 52 weeks after exposure.

Adamson, Correa, and Dalgard (1973) reported the induction of liver tumors in a rhesus monkey with aflatoxin B_1, and carcinogenicity of aflatoxin in the hamster has been described by Herrold (1969). Table 5.8 lists other species and target organs in which the tumorigenic activity of aflatoxin has been demonstrated.

[Although this report is primarily concerned with mammalian tests for carcinogenicity, Table 5.8 has been included because it concisely relates the association between exposure to a chemical or a chemical process and the occurrence of cancer in humans as reported by the International Agency for Research on Cancer (Tomatis et al., 1978). These data are correlated with the results of animal tests.]

5.2.2 Strain

The use of random-bred, rather than highly inbred, strains in chronic toxicity and carcinogenicity testing remains controversial. In broad-scale studies where little prior information exists, some workers

Table 5.8. Chemicals or industrial processes associated with cancer induction in humans: comparison of target organs and main routes of exposure in animals and humans

Chemical or industrial process	Humans			Animals		
	Main type of exposure[a]	Target organ	Main route of exposure[b,c]	Animal	Target organ	Route of exposure[c]
Aflatoxins	Environmental, occupational	Liver	p.o., i.h.[d]	Rat	Liver, stomach, colon, kidney	p.o.
				Fish, duck, marmoset, tree shrew, monkey	Liver	p.o.
				Rat	Liver, trachea	i.t.
					Liver	i.p.
				Mouse, rat	Local	s.c.
				Mouse	Lung	i.p.
4-Aminobiphenyl	Occupational	Bladder	i.h., s., p.o.	Mouse, rabbit, dog	Bladder	p.o.
				Newborn mouse	Liver	s.c.
				Rat	Mammary gland, intestine	s.c.
Arsenic compounds	Occupational, medicinal, and environmental	Skin, lung, liver[d]	i.h., p.o., s.	Mouse, rat, dog	Inadequate, negative	p.o.
				Mouse	Inadequate, negative	t., i.v.
Asbestos	Occupational	Lung, pleural cavity, gastrointestinal tract	i.h., p.o.	Mouse, rat, hamster, rabbit	Lung, pleura	i.h. or i.t.
				Rat, hamster	Local	ip..
				Rat	Local	i.p., s.c.
					Various sites[e]	p.o.
Auramine (manufacture of)	Occupational	Bladder	i.h., s., p.o.	Mouse, rat	Liver	p.o.
				Rabbit, dog	Negative	p.o.
				Rat	Local, liver, intestine	s.c.
Benzene	Occupational	Hemopoietic system	i.h., s.	Mouse	Inadequate	t., s.c.
Benzidine	Occupational	Bladder	i.h., s., p.o.	Mouse	Liver	s.c.
				Rat	Liver	p.o.
					Zymbal gland, liver, colon	s.c.
				Hamster	Liver	p.o.
				Dog	Bladder	p.o.
Bis(chloromethyl) ether	Occupational	Lung	i.h.	Mouse, rat	Lung, nasal cavity	i.h.
				Mouse	Skin	t.
					Local, lung	s.c.
				Rat	Local	s.c.

Table 5.8 (continued)

Chemical or industrial process	Humans			Animals		
	Main type of exposure[a]	Target organ	Main route of exposure[b]	Animal	Target organ	Route of exposure[c]
Cadmium-using industries (possibly cadmium oxide)	Occupational	Prostate, lung[d]	i.h., p.o.	Rat	Local, testis	s.c. or i.m.
Chloramphenicol	Medicinal	Hemopoietic system	p.o., i.j.		No adequate tests	
Chloromethyl methyl ether (possibly associated with bis(chloromethyl)-ether	Occupational	Lung	i.h.	Mouse	Initiator	s.
					Lung[d]	i.h.
					Local, lung[d]	s.c.
				Rat	Local[d]	s.c.
Chromium (chromate-producing industries)	Occupational	Lung, nasal cavities[d]	i.h.	Mouse, rat	Local	s.c., i.m.
				Rat	Lung	i.b. impl.
Cyclophosphamide	Medicinal	Bladder	p.o., i.j.	Mouse	Hemopoietic system, lung	i.p., s.c.
					Various sites	p.o.
				Rat	Bladder[d]	i.p.
					Mammary gland	i.p.
					Various sites	i.v.
Diethylstilbestrol	Medicinal	Uterus, vagina	p.o.	Mouse	Mammary	p.o.
				Mouse	Mammary, lymphoreticular, testis	s.c., s.c. impl.
					Vagina	Local
				Rat	Mammary, hypophysis[d] bladder	s.c. impl.
				Hamster	Kidney	s.c.; s.c. impl.
				Squirrel monkey	Uterine serosa	s.c. impl.
Hematite mining (? radon)	Occupational	Lung	i.h.	Mouse, hamster, guinea pig	Negative	i.h., i.t.
				Rat	Negative	s.c.
Isopropyl oils	Occupational	Nasal cavity, larynx	i.h.		No adequate tests	
Melphalan	Medicinal	Hemopoietic system	p.o., i.j.	Mouse	Initiator	s.
					Lung, lymphosarcomas	i.p.
				Rat	Local	i.p.

Table 5.8 (continued)

Chemical or industrial process	Humans			Animals		
	Main type of exposure[a]	Target organ	Main route of exposure[b]	Animal	Target organ	Route of exposure[c]
Mustard gas	Occupational	Lung, larynx	i.h.	Mouse	Lung Local, mammary	i.h., i.v. s.c.
2-Naphthylamine	Occupational	Bladder	i.h., s., p.o.	Hamster, dog, monkey Mouse Rat, rabbit	Bladder Liver, lung Inadequate	p.o. s.c. p.o.
Nickel (nickel refining)	Occupational	Nasal cavity, lung	i.h.	Rat Mouse, rat, hamster Mouse, rat	Lung Local Local	i.h. s.c., i.m. i.m. impl.
N,*N*-Bis(2-chloroethyl)2-naphthylamine	Medicinal	Bladder	p.o.	Mouse Rat	Lung Local	i.p. s.c.
Oxymetholone	Medicinal	Liver	p.o.		No adequate tests	
Phenacetin	Medicinal	Kidney	p.o.		No adequate tests[e]	
Phenytoin	Medicinal	Lymphoreticular tissues	p.o., i.j.	Mouse	Lymphoreticular tissues	p.o., i.p.
Soot, tars, and oils	Occupational, environmental	Lung, skin (scrotum)	i.h., s.	Mouse, rabbit	Skin	t.
Vinyl chloride	Occupational	Liver, brain[d], lung[d]	i.h., s.	Mouse, rat	Lung, liver, blood vessels, mammary, Zymbal gland, kidney	i.h.

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[a] The main types of exposures mentioned are those by which the association has been demonstrated; exposures other than those mentioned may also occur.

[b] The main routes of exposure given may not be the only ones by which such effects could occur.

[c] p.o. — peroral; i.t. — intratracheal; i.p. — intraperitoneal; s.c. — subcutaneous; t. — topical; i.v. — intravenous; ipl. — intrapleural; i.h. — inhalation; i.m. — intramuscular; s. — skin; s.c. impl. — subcutaneous implantation; i.b. — intrabronchial implantation; i.j. — injection.

[d] Indicative evidence.

[e] The induction of tumors of the nasal cavities in rats given phenacetin has been reported recently (S. Odashima, personal communication, 1977).

recommend using random-bred strains to ensure observing a species-characteristic response (Benitz, 1970). Other investigators urge the use of inbred animals whose genetic stability tends to reduce variations in the incidence of spontaneous disease and contributes to greater experimental precision (Food Safety Council, 1978; Womack, 1979). However, Greenman, Delongchamp, and Highman (1979) warn that easy generalization cannot be made about the variability of inbred and hybrid mice. Whether or not inbred or random-bred strains are used, it is important that background clinical and pathological information be available for test animals so that results in both control and treated animals can be compared with known literature values (Fancher, 1978; World Health Organization, 1978). Germ-free animals are rarely used in chronic toxicity and carcinogenicity testing because maintenance is too laborious and costly for long-term studies, but specific pathogen-free and gnotobiotic animals have been found useful by some investigators (Benitz, 1970).

Selection of the appropriate animal strain is an important part of experimental design in bioassays for carcinogenicity. In terms of numbers of strains available, selection is not easy. For example, there are over 250 mouse strains, 100 rat strains, and 30 hamster strains (Butler, 1979). Weisburger (1976) suggested several factors that deserve consideration in choosing a strain. These include maintenance requirements, sensitivity to test chemicals, and occurrence of diseases, both nonneoplastic and neoplastic. In addition, he reviewed information about some of the strains of rats, mice, and hamsters used in bioassays of carcinogenic agents. Because of variation in response to such agents by animals of different strains, it is generally advisable to test the agents in several strains of a species (Butler, 1979). Several studies will expand upon some of these considerations.

5.2.2.1 Hamsters and Guinea Pigs — Inbred strains of Syrian golden hamsters respond differently to subcutaneously injected methylcholanthrene and benzopyrene (Homburger et al., 1972). The less sensitive strains (BIO 82.73, BIO 1.5) have longer induction times than does the more sensitive 87.20 line. This fact is important for determining the duration of an experiment and, if not properly understood, could lead to misinterpretation of data. Another consideration in selection of an appropriate strain relates to tumor type. For example, some strains of hamsters (BIO 54.7, BIO 82.73, BIO 86.93) are not susceptible to induction of intestinal neoplasms and thus should not be used for bioassays concerned with such tumors (Homburger et al., 1972).

A strain may be sensitive to one chemical and not another, as seen in the Hartley and inbred ICRF guinea pigs. Diethylnitrosamine is an effective carcinogen in both strains; methylcholanthrene is inconsistent in the Hartley strain, but less so in the ICRF animals (Dale et al., 1973).

5.2.2.2 Mice — The influence of genetic factors has been clearly demonstrated in two strains of mice, BALB/c and C57BL/6, which after treatment with diethylstilbestrol developed testicular teratomas and pituitary neoplasms, respectively. The F_1 hybrids of these strains developed both tumors in response to the carcinogen (Greenman and Delongchamp, 1979). In addition to variation in tumor types among different strains, incidence, induction time, and susceptibility to an agent can vary. In an extensive study of transplacentally injected 1-ethyl-1-nitrosurea, Diwan and Meier (1974) demonstrated clear differences in all these parameters for five mouse strains (AKR/J, SWR/J, DBA/2J, C57BL/6J, and C57L/J). They concluded that responses to the carcinogen were dependent on both the strain involved and the fetal age at the time of injection. The latent period for malignant lymphoma in "101" mice treated with dimethylbenzanthracene (DMBA) was shorter than that of CBA mice (Roe, Rowsen, and Salaman, 1961). Thiery and van Gijsegem (1965) studied the induction of squamous cell carcinoma of the cervico-vaginal epithelium by 3,4-benzo(*a*)pyrene in 12 mouse strains. On the basis of tumor yield and induction time, strains C3H, A, 0.20, N, R, K, Q, and Mo were highly sensitive, S and AKR were moderately sensitive, and H and W were relatively insensitive to the carcinogen. Holland, Gosslee, and Williams (1979) demonstrated that C57BL/6 mice were 2.4 times more sensitive to the epidermal carcinogens bis(2,3-epoxycyclopentyl)ether, 2,2-bis(*p*-glycidyloxphenyl) propane, and *m*-phenylenediamine. Holland et al. also noted that the sensitivity of an assay could be affected by the choice of strain in the case of very weak carcinogens.

5.2.2.3 Rats — Within a few weeks after one dose of dimethylbenzanthracene, Sprague-Dawley rats developed high incidence of multiple mammary gland tumors. In contrast, Long-Evans rats receiving the same treatment had low incidence. few tumors, extended induction time, and, in some cases, tumor regression. The sensitivity of the mammary glands to the hydrocarbon can be correlated with inherited variation in pituitary function. Fibroadenoma incidences after DMBA in F_1 hybrids from reciprocal crosses of the two strains were nearly equal; hybrids from backcrosses in which the strain of origin was SD had high tumor incidence as opposed to low ones from the LE strain. Foster nursing had no effect on tumor incidence (Sydnor et al., 1962). Sydnor (1973) also tested nine inbred strains of rats for susceptibility to benzo(*a*)pyrene. Sarcoma incidence ranged from 38% in Long-Evans rats to 97% in Fischer rats. The most sensitive strains in terms of incidence and induction time were Sprague-Dawley, Fischer, and Werck-Stewart, and the least sensitive were the Long-Evans rats. Sydnor concluded that susceptibility is genetically determined by carcinogen dose.

In conclusion, careful selection of appropriate strains for experimentation and substance evaluations is a prerequisite for meaningful interpretation of data. Strain-related parameters that relate to carcinogenesis include spontaneous tumor incidence (discussed elsewhere), age, induction time, and susceptibility. Superimposed on the genetic framework are the many environmental factors that can modify the response of a strain to a particular stimulus. These factors must be controlled as extensively as possible. The great value of strain differences lies in the potential for eventually determining the molecular mechanisms that vary between strains. Ultimately, such knowledge may help to determine those mechanisms that cause variation among individuals.

5.2.3 Spontaneous Tumors

Spontaneous tumors are relatively common among experimental animals, both those bred for laboratory studies as well as wild animals maintained in the laboratory (Andervont and Dunn, 1962). Such lesions occur in a variety of organs and at different ages. Snell (1965) defined spontaneous lesions as those for which a cause cannot be ascertained. Such neoplastic lesions appear in animals that have not been experimentally exposed to a carcinogenic agent. Hoag (1963) pointed out that spontaneous tumors are generally considered "naturally occurring"; however, when the etiology of the particular tumor is understood, it can be induced experimentally. Spontaneous tumor incidences in the various organs of animals used in the National Cancer Institute Bioassay Program have been summarized by Page (1977*a*) and are listed in Table 5.9. The B6C3F$_1$ mouse, an F$_1$ hybrid cross between the C57BL/6 female and the C3H male, has a relatively high incidence of spontaneous tumors of the lung and liver. The F/344 rat has a high incidence of testicular tumors in the male and of pituitary tumors in the female. These strains have been used extensively. They have the advantages of good survival, disease resistance, and a relatively low spontaneous tumor incidence in organs other than those mentioned.

In order to make valid interpretations and comparisons of results in a variety of experimental situations -- whether they are designed to test carcinogenicity of specific agents, to screen substances for anti-carcinogenic activity, or to look at mechanisms of neoplastic growth -- the investigators must be cognizant of the incidence of spontaneous tumors in the animal tested. For example, the tumor response (i.e., the type, frequency, latency, etc.) to carcinogenic agents may vary among animals with different spontaneous tumor rates. For more information relating to this point, see the discussion of strain differences. Various factors can influence frequency, distribution, latency, and morphology of spontaneously occurring tumors. Some variables known to have effects are: breeding methods, genetics, age, diet, maintenance, histologic screening, and statistical evaluation (Pollard and Kajima, 1970; Pour et al., 1976; Sass et al., 1975), as well as geophysical parameters such as geography and climate (Gilbert et al., 1958). Sex and age incidence of characteristic tumors for certain inbred strains of mice have been summarized by Weisburger and Weisburger in Table 5.10. Spontaneous tumor incidence can generally be viewed as the result of both genetic and environmental influences whose relative contributions must be defined for a particular animal strain. Genetic or strain differences in spontaneous tumor frequency can be best evaluated when environmental factors have been characterized (Gilbert et al., 1958).

Table 5.9. Spontaneous tumor incidence in animals used in the National Cancer Institute Bioassay Program

Organ	B6C3F1 mouse[a]		Fischer 344 rat[a]		Osborne-Mendel rat[a]		Sprague-Dawley rat[b]		Charles River CD rat[b]	
	Males 1132[c]	Females 1176	Males 846	Females 840	Males 112	Females 149	Males 144	Females 145	Males 183	Females 184
Brain	<1		1.3	<1	<1		1.4		2.7	1.6
Skin/subcutaneous	1.0	<1	5.7	2.5	4.5	3.4	2.8	1.4	6.6	3.8
Mammary gland		<1	1.0	18.8	3.6	32.2	7.8	35.9	<1	45.1
Spleen	<1	<1	<1	<1	1.6				<1	
Lung/trachea	9.2	3.5	2.4	<1					1.6	3.8
Heart			1	<1		1.3				
Liver	15.7	2.5	1.2	1.3		1.3			1.1	2.2
Pancreas	<1	<1	<1	<1		<1	<1			
Stomach/intestines	1.3	<1	<1	1.0	<1				<1	<1
Kidney	<1	<1	<1	<1	6.3	3.4		<1	1.6	
Urinary bladder		<1	<1	<1			<1	<1	<1	
Testis	<1	NA[d]	76.2	NA		NA	2.8	NA	4.4	NA
Ovary	NA	<1	NA	<1	NA	2.0	NA		NA	1.1
Uterus	NA	1.9	NA	16.8	NA	4.0	NA	<1	NA	3.3
Pituitary	<1	3.5	10.2	29.5	8.0	15.4	11.1	32.4	33.3	57.6
Adrenal	<1	<1	8.7	4.0	11.6	2.0	1.4	2.8	7.7	4.3
Thyroid	1.1	<1	5.1	5.6	3.6	12.7		2.8	3.8	
Parathyroid									<1	
Pancreatic islets	<1	<1	3.2	1.3	2.7	1.3	<1		2.7	
Thymus	<1						<1			
Body cavities	<1	<1	<1	<1	<1		2.1	2.1	1.1	1.1
Leukemia/lymphoma	1.6	6.8	6.5	5.4	2.7	<1	<1		1.6	<1

[a] Studies terminated at 21 to 24 months.

[b] Studies terminated at 24 months.

[c] Number of animals necropsied.

[d] NA — not applicable.

Source: Adapted from Page, 1977a. Reprinted with permission of the publisher.

Table 5.10. Sex and age incidence of characteristic tumors of inbred strains

Tumor type and strain	Incidence (%)	Type mouse	Age (months)
Mammary gland tumor			
C3H	99	Breeding ♀♀	Av. 7.2
	100	Virgin ♀♀	Av. 8.8
DBA/2	77	Breeding ♀♀	Av. 15
	Lower	Virgin ♀♀	
A	84	Breeding ♀♀	
	5	Virgin ♀♀	
DD	84	Breeding ♀♀	Av. 7.7
	75	Virgin ♀♀	Av. 10.2
Lymphocytic leukemia			
AKR	92		Av. 8.0
C58	90		Av. 10.0
Primary lung tumor			
A	90		After 18
SWR	80		After 18
Hepatoma			
C3HeB	91	Breeding ♂♂	Av. 21.4
	58	Virgin ♀♀	Av. 24.1
	30	Breeding ♀♀	Av. 21.0
Reticulum cell neoplasm, type B SJL	91	Virgin ♀♀	Av. 13.3
	High	Breeding ♀♀, ♂♂	
Reticulum cell neoplasm, type A			
C57BL	15	Breeding ♀♀	Av. 22.3
Ovarian tumor			
C3HeB/De	47	Virgin ♀♀	Av. 24.3
	37	Breeding ♀♀	Av. 21.5
C3HeB/Fe	64	Breeding ♀♀	After 19
	22	Virgin ♀♀	After 19
RIII	60	Breeding ♀♀	After 17
	50	Virgin ♀♀	After 17
CE	34	♀♀	After 20
Pituitary tumor			
C57L	33	Breeding ♀♀	Old
C57BR/cd	33	Breeding ♀♀	Old
Hemangioendothelioma			
HR/De	24	♂♂ and ♀♀	Av. 22
Adrenal cortical tumor			
CE	100	Gonadectomized ♀♀	After 6
	79	Gonadectomized ♂♂	After 7

Table 5.10 (continued)

Tumor type and strain	Incidence (%)	Type mouse	Age (months)
Testicular teratoma			
129	1	♂♂	Congenital
	82	Male gonadal ridges transplanted to adult testes	
Myoepithelioma			
BALB/c	4		
A	Similar		
Skin papilloma			
HR/De	9	Hairless	Av. 22
Skin carcinoma			
HR/De	3	Hairless	Av. 18
Subcutaneous sarcoma			
C3H/J	3	♀♀	Av. 25
Harderian gland tumor			
C3H	1		Av. 20.7

Source: Weisburger and Weisburger, 1967, as adapted from Murphy, 1966. Reprinted with permission of the publisher.

Genetic control of susceptibility to ileocecal immunocytomas in certain strains of rats appears to reflect one or more dominant genes for susceptibility in the LOU/c strain and one or more loci of resistance in the OKA strain. F_1 progeny from crosses of these strains have null tumor incidence (Beckers and Bazin, 1978). High incidence of spontaneous congenital testicular teratomas in an inbred subline of mice is probably due to a single gene mutation (Stevens, 1973).

Evidence of genetic involvement at the molecular level is evident in the inhibition of mammary tumors in mice by treatment with a bacterial cell wall skeleton preparation which reduces the level of DNA synthesis in normal mammary glands and lowers circulating prolactin levels, both major factors in tumorigenesis (Nagasawa, Yanai, and Azuma, 1978).

Genetic influence on susceptibility has been demonstrated in certain strains of C3H mice prone to hepatomas and mammary tumors. The A^{vy} gene increases the incidence of both tumor types, perhaps by enhancing the virulence or transmission of the mammary tumor virus by either parent or by increasing the responsiveness of tissues. The gene's effects are also correlated with increased body weight and are enhanced by hormonal factors (Heston and Vlahakis, 1968; Vlahakis, Heston, and Smith, 1970).

Interesting results with this same C3H-A^{vy} system have been reported by Sabine, Horton, and Wicks (1973). Mice bred in Australia had 0% incidence of mammary tumors compared with the incidence of nearly 100% found in the United States. By using feed and bedding from the United States, the high tumor incidence can be restored. The interactions of genetic and environmental factors are clearly complex, and both deserve careful attention.

Examples of the modification of spontaneous tumor incidence by environmental factors have been reported in the C3H mouse hepatic tumor system, which can be influenced by sex, population density, and diet (level of protein and caloric intake) (Grahn and Hamilton, 1964; Heston, Vlahakis, and Deringer, 1960; Peraino, Fry, and Staffeldt, 1973; Silverstone and Tannenbaum, 1951; Tannenbaum and Silverstone, 1949). Heston (1958), in studying four substrains of C3H mice, found that mammary tumor incidence was influenced not only by genetic susceptibility, but also by hormonal stimulation during breeding and by age. Riley (1975) described a shortening of latent period for mammary tumors in C3H/He mice carrying the Bittner oncogenic virus and exposed to environmental stress factors.

Age of the animals and latency periods of the tumors can present problems in design and interpretation of experiments. Tumor incidence data from rats at 2 to 2.5 years of age could give a different picture from that which might be seen if the animals lived to old age. If the animals are more susceptible to tumors late in life, then testing only in young animals could prevent detection of neoplasms. Studies over the life span of the animal are necessary for the evaluation of the biologic behavior of spontaneous tumors and, possibly, of some carcinogen-induced tumors (Burek and Hollander, 1977). In addition to the value of life span data, one must consider the latency period before the effect of a carcinogen is detectable as well as the dose (which in some cases determines latency). Laboratory animals tend to be more susceptible to tumors as they age, but, as discussed earlier, additional factors are involved (Gilbert et al., 1958). Table 5.11 summarizes some pertinent literature on this subject.

5.2.4 Number

The number of animals used in chronic toxicity and carcinogenicity tests represents a compromise between the need for a sufficiently large number of animals to allow adequate statistical precision of the conclusions and the need to place a reasonable limit on costs and the experimental work load. For example, if the true frequency of a toxic effect in test animals is 5%, a test group numbering at least 58 must be used to discover the effect with a probability of $P = 0.05$, but 90 animals are needed at the $P = 0.01$ level of significance, and 134 animals must be used to achieve the $P = 0.001$ level of confidence (Barnes and Denz, 1954). Although high levels of statistical probability are obviously desirable, large increases in group size may diminish the thoroughness and care that are necessary for a successful completion of the study and may be counterproductive. In general, more useful information can be obtained by conducting thorough studies with a relatively small number of animals than by performing incomplete experiments using an excessive number of animals (Benitz, 1970).

Early investigators of chronic noncarcinogenic toxicity tended to use 10 or fewer test animals (rodents) per group (Barnes and Denz, 1954); later, groups of 20 to 30 rodents per sex and dose level were considered sufficient for practical purposes (Benitz, 1970). More recently, a minimum of 50 rodents of each species and sex has been considered necessary, especially in work subject to federal agency regulation (*Federal Register*, 1978, 1979; Food Safety Council, 1978; National Academy of Sciences, 1977). Fewer rodents per group may be used if the number of dose levels is increased; however, little current literature support exists for groups smaller than 20 animals.

Test groups are generally smaller than indicated in the previous paragraph when the test species are nonrodents. If dogs, cats, or nonhuman primates are used in chronic toxicity studies, a minimum of at least four animals per dose and sex is usually considered necessary

Table 5.11. Spontaneous tumors in laboratory animals

Animal	Tumor and organ	Incidence and age	Comments	Reference
Monkey				
Macaca philippinensis and *Macaca mulatta*	Lymphosarcoma (kidney) Hemangioendothelioma (vertebra) Meningiomatosis (lumbar cord) Lipoma (choroid plexus) Endotheliomatosis (lumbar cord)	Overall (0.08%) Prepubertal	12 000 monkeys (1800 of them cynomolgus) observed over 3.5 years. Also looked at granulomatous processes.	Jungherr, 1963
Mouse				
C3H/St	Mammary tumors	Incidence 87%	Studied tumor regression after 5-methyl cytidine.	Strong and Matsunaga, 1975
Nude mutant, most with BALB/c, some with C3H or C57/BL/6 backgrounds	None	9%, generally die at 2 to 3 months of age	11 000 mice studied from birth to 3 months.	Rygaard and Povlsen, 1974
Nude mutant from BALB/c backgrounds	Lymphoreticular neoplasms	(Estimated from graphic material. Tumor incidence for entire group was about 30% at 77 weeks-42% at 85 weeks.)	Germfree conditions. Can maintain for over 20 months. In germfree nu/+ females, no mammary tumors seen.	Outzen et al., 1975
CD8F, (BALB/cXDBA/8F1)	Adenocarcinoma mammary (also metastasis to lung)	80%, 10 months	Studied chemotherapeutic activity.	Fugmann et al., 1977
SHN	Mammary tumors	41.3% at 7 months, 87.5% at 12 months	Studied tumor suppression.	Nagasawa, Yanai, and Azuma, 1978
129/terS	Teratomas, testicular	30%, earliest seen were in 16-day fetuses	High incidence probably due to a single gene mutation.	Stevens, 1973
Strain A	Lung tumors	0% at 3 months, 40% at 12 months, 77.1% at 18 months	Frequency of lung tumors has been reproducible for more than 30 years.	Shimkin and Stoner, 1975
CD$^{(R)}$-1HaM/ICR	Lymphoreticular mammary, pulmonary, osteogenic sarcoma, hemangiosarcoma, renal and hepatic tumors	Total incidence of tumors range of 0.77-50% for age groups ranging from 0+ to 20+ months	Percentages of a particular tumor type varied with age.	Percy and Jonas, 1971
C3H-A^{vy}, C3H-A^{vy}fB	Liver, mammary tumors	90-100% or 0%, ages from 7 to 22 months	Tumor incidence apparently related to type bedding used.	Sabine, Horton, and Wicks, 1973

Table 5.11 (continued)

Animal	Tumor and organ	Incidence and age	Comments	Reference
C3Hf	Mammary tumors: adeno-carcinoma,	22%, 20 months	Deprived of mammary tumor agent by foster nursing.	Heston, 1958
C3Hff	Adenoacanthoma, carcino-sarcoma	29%, 19 months		
C3Hf/2		51%, 20 months		
C3Hf/An		27%, 20 months		
C3HfB X YBR	Hepatoma	100%	Genotypes: $A^{Y}A$ males	Heston and Vlahakis, 1966
C3HfB X YBR		94%	Aa males	
C3Hfb X YBR		63%	$A^{Y}A$ females	
YBR X C3HfB		5%	$A^{Y}A$ males	
YBR X C3HfB		95%	Aa males	
YBR X C3HfB		43%	$A^{Y}A$ females	
YBR X C3HfB		41%	Aa females	
		0%	Weight and growth factors considered important.	
C3H/St	Adenocarcinoma, mammary	41%, 4.5 months	Study of effects of As and Se on tumor incidence.	Schrauzer et al., 1978
C3HX101 hybrids	Osteosarcomas	20%		
A	Lung tumors	71%, 23 months	Authors cited data of Bentvelzen and Szalay, 1966.	Shimkin and Stoner, 1975
GR		42%, 14 months		
020		58%, 21 months		
CBA		18%, 27 months		
C3H		14%, 21 months		
DBA		9%, 21 months		
C57BL		7%, 22 months		
NMRI-Neuherberg	Lymphoma	41%		Luz, 1977
	Lung tumors	16%		
	Glomerulosclerosis	13% (50% survival time		
	Ovarian neoplasms	12% is 600-700 days)		
	Adenomatous hyperplasia of the glandular stomach	7%		
Wild house mice (*Mus musculus*)	Pulmonary, reticulem-cell (type B), granulosa-cell of the ovary, hepatoma, hemangioendothelioma, mammary, lymphocytic neoplasm	36%, 2-24 months 64%, 25-33 months	Tumors in mice received in 1951 and their descendants until 1961.	Andervont and Dunn, 1962

Table 5.11 (continued)

Animal	Tumor and organ	Incidence and age	Comments	Reference
C3Hf/Anl(Anl 70)	Liver tumors	Females 6-13%, males 41-68%, before 12 months	Studied enhancement of tumor incidence.	Peraino, Fry, and Staffeldt, 1973
C3H/HeN+	Mammary tumors	99%, 7.2 months	Demonstration of mammary tumor virus antibodies in different strains. Highest titers in tumor bearing animals.	Ihle, Authur, and Fine, 1976
GR/N		70%, 10 months		
BALB/cfC3H				
C57BL/oXC3HF$_1$ (called B6C3F$_1$)		<10%, 8 months		
AKR		0%, 8 months		
BALB/c		3%, 15 months		
C57BL/6		1%, 15 months		
C3H/HeN		5%, 8 months		
C3H/HeJ C3H/He	Mammary tumors	27%, duration of experiment, 2 years. (In another table, incidence appears to be 100%.)	Reduction of tumor incidence by manipulation of estrogen and prolactin interactions with administration of 2 bromo-α-ergocryptine, 17β-estradiol, or Enovid.	Welsch, 1976
C3H/He	Mammary tumors	80-100% within 8 to 18 months	Strain carrying the Bittner oncogenic virus incidence greater under chronic stress.	Riley, 1975
C3H/A/BOM	Hepatoma	66%, 22-25 months, controls received rat albumin starting at 9 months. 50%, 18-20 months, controls received adjuvant in PBS starting at 2 months.	Study of increased levels of serum alpha-fetoprotein in mice with spontaneous liver-cell cancer.	Jalanko et al., 1978
C3HeB/Fej		25%, 12-16 months, control received adjuvant in PBS		
C3H	Hepatoma	85%	In males.	Heston, Vlahakis, and Deringer, 1960
C3Hf		72% All killed at 14 months	C3Hf male fed Purina chow instead of the NCI pellets had a 57% incidence.	
C3He		78%		
C3H-A^{vy}	Hepatoma	100%, 12 months	Males (also nearly all females at 16 months).	Heston and Vlahakis, 1968
	Mammary tumors	100%, 6-7 months	Females with MTV.	
C3H-A^{vy}fB	Mammary tumors	90%, 15 months	Female breeders free of MTV but having NIV.	
		20%, 15 months	Female virgins free of MTV but having NIV.	

Table 5.11 (continued)

Animal	Tumor and organ	Incidence and age	Comments	Reference
Hamster				
Syrian, Eppley colony, Hannover colony	Overall tumor incidence Primary tumors in respiratory, digestive, urogenital, endocrine, vascular, lymphatic systems	32%, average 41% survival time for animals with multiple tumors was 97 and 85 weeks, respectively	Study of spontaneous tumors and diseases in Syrian hamster colonies.	Pour et al., 1976
BIO 4.24	Adrenal tumors	52%, average age at autopsy, 80.3 weeks		Homburger and Russfield, 1970
BIO 45.5		17%		
Rat				
CD CD-1	Sarcoma, fibroma, adenocarcinoma, adenoma, hemangioma, fibroadenoma, adenoma, leiomyoma, neck, jaw, flank, shoulder, salivary gland, pituitary gland, thyroid gland, spleen, mammary gland, uterus	25%, weeks of observation of tumors ranged from 23 to 80 weeks	Controls in study of oral toxicity of organic fungicides.	Lee, Russell, and Minor, 1978
BN/BiRij	Papillary tumors - urinary bladder Squamous cell carcinomas - ureter	Males 28% (bladder), 6% (ureter), ages 7-48 months Females 2% (bladder), 54% (ureter), ages 7-54 months	First report of high incidence of such tumors in rats. These spontaneous tumors are rare.	Boorman and Hollander, 1974
LOU/C	Ileocecal immunocytomas Ileocecal lymph node for most	24%	Tumor incidence not homogeneous - could get wide variation among litters of the same strain.	Beckers and Bazin, 1978
LOU/M		1.7%		
AXC		2.6% 8-12 months		
OKA		0%		
(LOU/CXAXC) F_1		16%		
(LOU/CXAUG)F_1		12%		
(LOU/CXOKA)F_1		0%		
GG	Phaeochromocytoma (adrenal gland) the major tumor. Numerous others also occurred in breast, pituitary gland, thyroid gland, testis, pancreas, liver, kidney, ovary, salivary glands, uterus, and meninges	74% of all males, 50% of all females	Tumor incidence influenced by diet through effects on endocrine glands. used other strains also.	Gilbert et al., 1958
NB	Adrenal carcinoma	Females about 5% at 12+ months	Spontaneous tumors seldom found in males.	Noble, Hochachka, and King, 1975
	Breast fibroadenoma	About 20% at 12+ months		

Table 5.11 (continued)

Animal	Tumor and organ	Incidence and age	Comments	Reference
BDX	Sarcomas - connective tissue, bone, neural system, skin carcinomas, tumors of lung, gastrointestinal tract, genito-urinary tract, mammary glands, testis, adrenal glands; Malignancies of lymphoreticular system	66%, 13-30 months	Tumors were macroscopic, malignant as proven by histology and transplantation.	Zoller, Matzku, and Goerttler, 1978
Wistar - Af/Han-EMD	Tumors of the central nervous system: oligodendroglioma, astrocytoma, mixed glioma, pleomorphic glioma, meningioma	5.8%, mean ages ranged from 796 to 963 days		Sumi et al., 1976
BN/Bi	Mammary gland - fibroadenoma	11%, 30 months (23-40)	Data for other tumor types included. Study of incidence of benign and malignant neoplasms at different ages.	Burek and Hollander, 1977
	Pituitary tumors	Males, 16%, 28 months (15-42) Females, 26%, 31 months (17-39)		
	Lymphoreticular sarcomas	Males, 14%, 24 months (19-36)		
	Adrenal gland, cortical adenoma	Males, 12%, 33 months (27-43) Females, 19%, 33 months (23-54)		
	Pancreas - islet adenoma	Males, 15%, 33 months (20-42) Females, 11%, 33 months (23-40)		
	Cervix and vagina, sarcoma	15%, 27 months (18-38)		
ACI/N	Testes-interstitial cell tumors, adrenal gland, pituitary gland, urinary bladder, mammary gland, thymus, lymph nodes, subcutaneous tissue, heart, vagina, salivary gland, others	56% in males 52% in females Developed in 169 weeks		Maekawa and Odashima, 1975

(National Academy of Sciences, 1977); however, currently proposed U.S. Environmental Protection Agency regulations require six nonrodent animals per exposure group (*Federal Register*, 1979). This number does not include allowances for interim sacrifices and must be increased if that practice is followed. Needless to say, toxic reactions with low incidence rates are unlikely to be detected with such small groups (Barnes and Denz, 1954). Consequently, nonrodent test animals are usually used only as "second" species in conjunction with larger numbers of rodent test animals.

For carcinogenicity testing, the number of animals (rodents) to be used is influenced by two additional factors not pertinent to chronic toxicity evaluations (Magee, 1970; Page, 1977*b*). First, a significant number of animals must survive to tumor-bearing age. Second, the incidence of spontaneous tumors in the control groups must be compensated for in order to avoid jeopardizing the sensitivity of the test. Table 5.12 shows, in relation to animal numbers, how such an increase in spontaneous tumors in the controls will affect the incidence of observed tumors required for significance. Experience has shown that in most general carcinogenicity tests, 50 animals per sex per dose level will be sufficient to meet these two requirements (National Academy of Sciences, 1977; Page, 1977*b*; Sontag, Page, and Saffiotti, 1976). However, in any case, the number surviving to tumor-bearing age should be at least 25 per sex per level (World Health Organization, 1978).

5.2.5 Controls

Each chronic toxicity or carcinogenicity test should be evaluated with reference to a concurrent control group composed of animals of the same species, age, sex, and weight as the treated groups (*Federal Register*, 1979; U.S. Food and Drug Administration, 1971; Weil, 1962; Weil and Carpenter, 1969). These animals should be handled identically to the test species except for treatment with the test material (World Health Organization, 1978). Most investigators require that this negative control group contain at least as many animals of each sex as the test groups; other workers recommend that it contain twice as many (National Academy of Sciences, 1977). In lieu of a negative control group, a

Table 5.12. Incidence of tumors in treated groups required for significance ($P = 0.05$) depending on experimental group size and spontaneous tumors in controls[a]

Incidence of tumors in controls (%)	Number of animals per group[b]				
	10	25	50	75	100
0	50	20	12	8	6
10	70	40	28	24	21
20	80	52	40	36	34
30	90	64	52	47	45
40	100	72	62	58	55

[a] Calculations based on tabulations of Mainland and Murray, 1952, as cited in Page, 1977*b*.

[b] Controls and treated groups of same size.

Source: Adapted from Page, 1977*b*. Reprinted with permission of the publisher.

vehicle control group is often required when the test material is administered by gavage (World Health Organization, 1978). If the toxic properties of the vehicle are not well documented, both negative and vehicle control groups may be recommended (*Federal Register*, 1979; Page, 1977*b*).

The use of "positive controls," animals treated with a chemical of known toxic or carcinogenic potential utilizing the same or a very similar test design, is occasionally recommended for chronic toxicity or carcinogenicity tests (Page, 1977*b*). In chronic tests, its usefulness is not well established (Benitz, 1970), and it is only infrequently suggested in order to detect borderline effects and minimal enhancement of normal pathological or age-related conditions (*Federal Register*, 1979; National Academy of Sciences, 1977).

For carcinogenicity tests, positive controls can provide more information, including: (1) establishing the test animals' ability to respond to a carcinogenic insult, (2) helping detect genetic drift; (3) providing baseline information on new test species or strains; (4) revealing any accidental inclusion of extraneous factors that could affect the test; (5) providing an indirect evaluation of the laboratory and its general bioassay program; and (6) assessing the relative carcinogenic potential of the test chemical (Food Safety Council, 1978; Page, 1977*b*; World Health Organization, 1978). Despite all this potential information, the applicability of positive controls is limited. Most discussions suggest that it be applied only to series of analogous chemicals and need not be done with every study (Food Safety Council, 1978; Page, 1977*b*; Peck, 1974; U.S. Food and Drug Administration, 1971).

5.2.6 Age

The age at which animals are started on test is an important and sometimes overlooked consideration in testing for chronic toxicity and carcinogenicity. Because it is a basic principle of chronic toxicity and carcinogenicity testing that test animals should be exposed to the test material for a major portion of their life span, it is generally agreed that animals should be put on test at the earliest practical age (National Academy of Sciences, 1977). In most published screening studies for carcinogenesis, weanling or immediately postweanling rats and mice have been used (Loomis, 1974; Magee, 1970; Ministry of Health and Welfare Canada, 1975; National Academy of Sciences, 1977; World Health Organization, 1978). Effects of the age of the test animal on its response to carcinogenic challenge are well documented, and the remainder of this section will be devoted to a discussion of these effects.

The use of weanlings or immediately postweanlings permits testing under a variety of conditions not present in mature animals (e.g., during periods of active protein synthesis, cellular proliferation, and sexual maturation). In all of these physiological stages the weanling's response to carcinogens is expected to be more acute than that of adults (Weisburger and Weisburger, 1967).

Numerous studies have demonstrated that infant, newborn, and even in utero rodents may have greatly increased susceptibilities to certain carcinogens compared with weanlings. For example, Pietra, Spencer, and Shubik (1959) observed a 32% incidence of malignant lymphomas in 11- to 24-week-old Swiss mice following a single 30-µg subcutaneous dose of

9,10-dimethyl-1,2-benzanthracene administered at age 12 h or less. Similarly, Kelly and O'Gara (1961) induced pulmonary tumors in essentially all 18-h-old albino mice treated subcutaneously with 0.02 mg dibenz(*a*,*h*)-anthracene or 0.011 mg 3-methylcholanthrene.

Many other workers have also demonstrated the extraordinary sensitivity of newborn mice to certain carcinogens (Axelrad and van der Gaag, 1962; Berenblum, Boiato, and Trainin, 1966; Chieco-Bianchi et al., 1963; De Benedictis et al., 1962; Doell and Carnes, 1962; Flaks, 1965, 1966; Gargus, Paynter, and Reese, 1969; Kaye and Trainin, 1966; Klein, 1963; Liebelt, Liebelt, and Lane, 1964; Liebelt, Yoshida, and Gray, 1961; Nishizuka, Nakakuki, and Sakakura, 1964; Roe, Mitchley, and Walters, 1963; Toth, Magee, and Shubik, 1964; Toth, Rappaport, and Shubik, 1963; Vesselinovitch and Mihailovich, 1966; Vesselinovitch, Milhailovitch, and Itze, 1970; Vesselinovitch et al., 1972, 1975; Vesselinovitch, Rao, and Milhailovitch, 1975). Similar and related studies have been performed with hamsters (Lee, Toth, and Shubik, 1963) and rats (Baba and Takayama 1961; Howell, 1963; Toth and Shubik, 1963). Many of these studies were critically reviewed by Toth (1968) or Della Porta (1968).

In view of the apparently accentuated sensitivity of newborn animals to selected carcinogens, some early workers believed that a relatively quick and sensitive screening test for carcinogens might be achieved by exposing newborn animals to single doses of the test material (Della Porta and Terracini, 1969; Epstein, Andrea, and Jaffe, 1967; Gorrod, Carter, and Roe, 1968; Roe, Carter, and Adamthwaite, 1969; Roe, Rowsen, and Salaman, 1961; Toth, 1968). However, subsequent research indicated such exposures could be more complex than they first appeared. For example, treatment with *N*-hydroxy-*N*-2-fluorenylacetamide produced no detectable tumors in infant rats but did induce tumors in weanlings under similar conditions (Weisburger et al., 1970). Also, dimethylnitrosamine induced renal adenomas in mice exposed as adults, but not in those exposed as newborns (Terracini et al., 1966). In retrospect, it became apparent that since newborns differ from weanlings or adult animals in metabolic capability, viral susceptibility, and hormonal status, as well as other anatomical and physiological characteristics, it is illogical to expect identical responses from each of these age groups (Ministry of Health and Welfare Canada, 1975; Rice, 1976). It is reasonable to expect newborns to respond more sensitively than adults to ultimate carcinogens because of reduced immunological and hormonal competence. But because of the lack of well-developed metabolic capabilities, responses of such animals to proximate carcinogens that require metabolic activation will obviously be deficient or absent compared with more mature animals (Page, 1977*b*). It is thus clear that negative results from tests involving newborn animals cannot be the sole basis of evaluating the carcinogenicity of a test material. Indeed, Roe (1975) even questioned the wisdom of accepting positive results from such experiments.

In principle, test sensitivity and age-related effects should be maximized if animals are exposed to the test material during all prenatal and postnatal phases of their lives (Munro, 1977; Page, 1977*b*; U.S. Food and Drug Administration, 1971). Such a procedure requires treatment of the pregnant mother and continuing postnatal exposure of the offspring. Such a bigenerational exposure was strongly recommended as a routine approach to carcinogenesis testing by several national and international authorities following the discovery of vaginal adenocarcinomas in daughters of women who had received diethylstilbestrol during pregnancy (Greenwald et al., 1971; Herbst, Ulfelder, and Poscanzer, 1971; Munro, 1977). Furthermore, several subsequent studies showed that prenatal exposures to certain known carcinogens in fact result in high incidences of tumors in offspring (Andrianova, 1971; Goerttler and Lohrke, 1977; Mohr, 1973; Spatz and Laqueur, 1967; Svenberg et al., 1972; Tomatis et al., 1971; Turusov et al., 1973). Ivankovic (1973) reported transplacental induction of tumors in rats with ethylnitrosourea. Five to eighty mg per kg of body weight of the compound given as a single dose, intravenously or orally, on day 13 to 23 of pregnancy produced a significant yield of tumors of the nervous system in the offspring. However the 5-mg/kg dose was ineffective in adult rats observed throughout their entire life span. In fact, a notable yield of tumors in the nervous system of adults was obtained only when 140 to 200 mg/kg were given, thus demonstrating increased sensitivity of the fetus to carcinogenesis.

However, routine testing for carcinogenesis through prenatal and bigenerational exposures is complicated by several factors that have not yet been adequately resolved. For example, dose determination and regulation for animals in utero is uncertain, and tolerances are variable and age dependent (Vesselinovitch, 1973). Also, many substances likely to be tested as carcinogens are teratogenic and may jeopardize the outcome of the cancer bioassay through reproductive deficiencies unless doses are carefully selected and timely administered. Furthermore, certain classes of compounds likely to be tested for carcinogenicity, such as chlorinated aliphatic and aromatic hydrocarbons, tend to accumulate in body fat and liver and may interfere with normal reproductive processes even though they are not strongly teratogenic. In an effort to minimize such reproductive interferences, some authorities recommend limiting in utero exposures to the second half of pregnancy when organogenesis is complete (Golberg, 1974); otherwise, teratogenicity studies would have to be done and nonteratogenic doses would have to be used for in utero studies. However, no generally accepted guidelines exist with respect to in utero and bigenerational exposures for carcinogenesis testing (Munro, 1977). Except for special studies, such as effects of certain food additives, pesticides, and drugs on pregnant women (U.S. Food and Drug Administration, 1971), bigenerational and in utero exposures are not generally made a part of routine carcinogenesis testing today.

5.2.7 Sex

Current practice calls for the use of equal numbers of male and female test animals in assays for chronic toxicity (*Federal Register*, 1978; Food Safety Council, 1978; National Academy of Sciences, 1977; World Health Organization, 1978), although early workers questioned the need for equity (Barnes and Denz, 1954). Essentially all test guidelines require that both sexes of the test species be used in carcinogenicity assays (Page, 1977*b*). However, female mice are preferred by some researchers because they are less aggressive and survive better than males; in some instances, male animals may be preferred when females are needed for breeding. In the following section, data will be presented that illustrate sex-related differences in the response of animals to chemical carcinogens and the effects certain intrinsic and extrinsic factors of the animal test system have on these differences. Comparisons will be made of the responses of male and female animals to chemicals within selected chemical classes: polycyclic hydrocarbons, nitroso compounds and amines, and halogenated hydrocarbons.

Akamatsu and Barton (1974) compared the neoplastic response of both sexes of five strains of mice that had received 1 mg of 3-methylcholanthrene intragastrically. Tumor incidence varied with strain and, in some cases, sex. Sixty-seven percent of the males of the BTO strain developed gastric tumors while only 30% of the females did, and more males (25%) of the C3H/HeOs strain had gastric tumors than did the females (12%). The C57BL/60s females had an increased incidence of lymphomas, 25%, while only 11% of the treated males responded. More females of the BALB/cOs strain developed pulmonary tumors (62%) than did the males (50%); methylcholanthrene-treated males in all five strains developed amyloidosis more often than did the females. Females of two strains of mice (BTOs and C3H/HeOs) had a high incidence of spontaneous mammary tumors, which was not increased by methylcholanthrene; however, strains with low mammary tumor incidence (BALB/cOs and C3H/HeOs), treated with methylcholanthrene, developed statistically significantly more mammary tumors than did their untreated controls. The C57BL/60s females, and males of all strains, did not develop any mammary tumors. The data (Table 5.13) in this experiment suggest that differences in tumor response of mice to systemic administration of methylcholanthrene are sex related and that sex-related differences are strain dependent.

Wistar rats given daily intragastric instillations of 3-methylcholanthrene for 9 weeks (for a total of 270 mg) also displayed distinct, sex-related differences in neoplastic responses (Gruenstein, Meranze, and Shimkin, 1966). Results of the study are summarized in Table 5.14. Of 122 treated females, 115 (94%) developed 120 total neoplasms. Of these, 117 were malignant. Of 81 treated males, 23 (28%) developed 26 malignant neoplasms. The difference in the tumor incidences of male and female rats can be accounted for mainly by the difference in the numbers of mammary carcinomas (87% of the females, 12.4% of the males). However, 16% of the males developed benign and malignant cutaneous tumors, while

Table 5.13. Systemic tumor induction in mice with 3-methylcholanthrene by the intragastric route

Strain	Number of animals	Tumor incidence (%)									
		Gastric	Skin	Lymphomas	Hepatomas	Pulmonary tumor	Bladder tumor	Mammary tumor	Ovarian tumor	Amyloidosis	Mice with tumors
BIO											
Treated[a] male	51	67	4	16	4	4	4			28	67
Untreated[b] male	93	0	2	4	15	2	0			19	19
Treated female	20	80	0	0	0	10	0	85	0	25	95
Untreated female	174	0	1	4	1	4	0	70	2	2	76
C57Bl/60											
Treated male	85	15	5	33	5	2	0			13	35
Untreated male	90	0	0	6	3	10	0			2	7
Treated female	20	20	5	25	0	5	0	20	0	10	45
Untreated female	50	0	0	1	0	0	0	0	0	3	3
BALB/c0											
Treated male	36	11	3	20	3	50	0			6	50
Untreated male	18	0	0	6	0	0	0			0	11
Treated female	42	14	1	33	5	62	0	24	5	0	81
Untreated female	104	1	4	13	2	1	0	32	4	0	36
C3HeB/0											
Treated male	70	34	[illegible]	3	3	1	0			11	29
Untreated male	142	0	0	5	18	[illegible]	0			0	22
Treated female	32	38	6	3	0	6	0	6	11	0	56
Untreated female	193	0	0	6	0	1	0	–	–	0	11
C3H/He0											
Treated male	24	23	9	4	4	4	0			4	46
Untreated male	253	0	0	0	14	0	0			0	34
Treated female	43	12	0	9	0	0	0	93	0	0	96
Untreated female	166	0	0	1	0	0	0	96	0	0	96

[a] All treated animals received 1 mg 3-methylcholanthrene in olive oil.

[b] Controls received olive oil alone.

Source: Adapted from Akamatsu and [illegible], 1974.

Table 5.14. Influence of sex on response to carcinogens

Species	Age at start of experiment	Chemical	Dose	Route	Tumor type	Tumor incidence		Reference
						Male	Female	
Wistar rat	6 weeks	3-Methylcholanthrene	270 mg (total)	Intragastric instillation	Sebaceous gland Cutaneous	18% 16%	0.8% 0.8%	Gruenstein, Meranze, and Shimkin, 1966
Fischer rat	3-6 months	3-Methylcholanthrene	1.9 mg/week	Skin painting	Skin	No difference		Zackheim, 1964
Lister rat	2-3 months	Dimethylbenzanthracene	0.05 ml { 0.5% 1.0% 2.0%	Salivary gland	Carcinoma	16% 75% 83%	0% 33% 21%	Glucksmann and Cherry, 1971
			0.05 ml { 0.5% 1.0% 2.0%	Salivary gland	Sarcoma	91% 25% 23%	49% 20% 100%	
B6C3F$_1$ mouse	1 day 15 days 42 days	Benzo(a)pyrene	75 μg/g body weight	Intraperitoneal	Liver	52% 60% 13%	2% 7% 0%	Vesselinovitch et al., 1975
	1 day 15 days 42 days	Benzo(a)pyrene	150 μg/g body weight	Intraperitoneal	Liver	81% 58% 9%	18% 7% 0%	
C3AF$_1$ mouse	1 day 15 days 42 days	Benzo(a)pyrene	75 μg/g body weight	Intraperitoneal	Liver	34% 27% 0%	2% 2% 0%	
	1 day 15 days 42 days	Benzo(a)pyrene	150 μg/g body weight	Intraperitoneal	Liver	46% 23% 3%	2% 2% 0%	
Buffalo rat	4 weeks 12 weeks 24 weeks 52 weeks	Diethylnitrosamine	0.0114%	Diet for 26 weeks	Total	60% 54% 33% 0%	21% 38% 23% 0%	Reuber, 1976
C3H/HeN mouse	8 weeks	4-Hydroxyaminoquinoline 1-oxide (4HAQO)	1 mg	Subcutaneous	Sarcoma	40%	20%	Shirasu, 1963
C57BL/6JN mouse	8 weeks	4-HAQO	1 mg	Subcutaneous	Sarcoma	10%	10%	
Albino mouse	8 weeks	4HAQO	1 mg	Subcutaneous	Sarcoma	11%	9%	

Table 5.14 (continued)

Species	Age at start of experiment	Chemical	Dose	Route	Tumor type	Tumor incidence		Reference
						Male	Female	
Holtzman		Ethionine		Diet for 7.5 months	Liver	60%	25%	Farber, 1963
Fischer rat		Ethionine		Diet for 7.5 months	Liver	100%	90%	
Wistar rat		Ethionine		Diet for 7.5 months	Liver	100%	100%	
B6C3F$_1$ mouse	6 weeks	Benzidene hydrochloride	30 ppm 150 ppm	Diet Diet	Liver Liver	6% 44%	26% 94%	Vesselinovitch, Rao, and Mihailovitch, 1975
Osborne-Mendel rat	3-4 months	4-Dimethylaminobenzene-1-azo-1-naphthalene	0.075%	Diet	Liver	75%	17%	Mulay and O'Gara, 1959
Osborne-Mendel rat	3-4 months	4-Dimethylaminoazobenzene	0.060%	Diet	Liver	85%	19%	
Osborne-Mendel rat	3-4 months	4-Dimethylaminobenzene-1-azo-2-naphthalene	0.075%	Diet	Liver	98%	100%	
Wistar rat	12 hours	4-Dimethylaminoazobenzene	1.2 mg	Subcutaneous	Liver	No difference		Baba and Takayama, 1961
ACX rat Intact Castrate Castrate-hormone	2-4 months	2-Diacetylaminofluorene	166-217 mg/kg body weight	Diet	Liver	60% 33% 29%	12.5% 0% 86%	Morris and Ferminger, 1956

the incidence of those tumors in females was less than 1%, and male rats developed more sebaceous gland tumors (18% incidence) than females (<0.8%). Other types of tumors were induced in this study, but no other sex differences were obvious in the responses. The cutaneous tumor data were at variance with the results of an earlier experiment in which Zackheim (1964) studied the cutaneous response of several strains of rats to skin painting with methylcholanthrene, anthramine, and 7,12-dimethyl(*a*)benzanthracene. Although he reported differences in types of skin tumor that were related to the chemicals administered, no significant strain or sex-related differences were observed. Gruenstein et al. suggested that the sex differences seen in their experiment were influenced by route of administration. In Zackheim's experiment the route was external (skin painting), whereas the route used in the Gruenstein et al. study was internal (intragastric).

Glucksmann and Cherry (1971) reported that, when low concentrations of 0.5% or 1.0% 9,10-dimethyl-1,2-benzanthracene were injected into rats locally in the salivary gland, about twice as many sarcomas and carcinomas were induced in males as in females (Table 5.14). These effects were confirmed when the administration of female hormones, estrogens, to males reduced the number of dimethylbenzanthracene-induced tumors by one-half and the administration of a male hormone, testosterone, doubled the number of tumors in carcinogen-exposed females. However, when 2% dimethylbenzanthracene was injected, the sex difference disappeared, suggesting that the sex differences observed were related to carcinogen dose and were therefore quantitative rather than qualitative.

In an experiment designed to test the effects of age, sex, and strain on tumor induction with benzo(*a*)pyrene, two strains of mice of various ages were injected intraperitoneally, with one dose of the carcinogen per mouse (75 or 150 µg per g of body weight) (Vesselinovitch et al., 1975). The life span study revealed specific effects associated with each of the modifiers (Table 5.14). Sex-influenced differences were most prominent in induction of liver and lymphoreticular tumors. Male rats of both strains developed significantly more liver tumors than females, whereas females of both strains developed more lymphoreticular tumors than the males. As the animals grew older, the response of the liver to the carcinogen decreased, the differential between the two sexes decreased, and the sex response therefore appeared to be related to age.

Reuber (1976) examined the effects of age and sex on the induction of preneoplastic and neoplastic lesions in the esophagus of Buffalo strain rats with diethyinitrosamine. Starting at 4, 12, 24, and 52 weeks of age, the animals were fed a diet containing 0.0114% diethylnitrosamine for 26 weeks. At 26 weeks, male rats that had been 4 weeks old when the treatment was started had a higher incidence of carcinomas of the esophagus (60%) than females of the same age group (21%). Among animals that began receiving treatment at 12 weeks of age, the difference between the sexes was slight by 28 weeks; in those that received the first treatment at 24 weeks, tumor incidence between the two sexes was practically the same by 36 weeks. The differences in the responses of the males and females diminished with increasing age. This finding supports the previously cited data of Vesselinovitch et al. (1975). See Table 5.14.

As in the experiments of Akamatsu and Barton (1974) with methylcholanthrene, the strain of test species was shown by Shirasu (1965) to influence the difference in sex response of mice to 4-hydroxyaminoquinoline-1-oxide. Results of the experiment are summarized in Table 5.14. Nine months after starting subcutaneous injection of 1 mg of the carcinogen (divided into 10 weekly injections), 40% of the male mice of the C3H/HeN strain had developed sarcomas at the injection site, while only 20% of the females developed the same type of tumor. Other strains tested developed sarcomas with incidences of 9% to 40%, but no sex-related differences were seen. Papilloma development appeared to be sex related in the C3H/HeN strain (papillomas in 40% of the females and 0% of the males), in the C57BL/6JN strain (papillomas in 0% of the females and 20% of the males), and in a noninbred albino strain (papillomas in 9.1% of the females and 0% of the males). None of the females and only 10% of the males of the C57BL/6JN strain had leukemia. Subcutaneous injection of 4-nitroquinoline-1-oxide produced sarcomas in 10% of the C3H/HeN males but none in the females.

One other experiment that showed sex-related differences in response to amino compounds was that of Farber (1963), who observed the response of Holtzman rats to hepatocarcinogenesis with ethionine. Feeding the chemical in the diet for 7.5 months produced liver cancer in 60% of the males and in 25% of the females. Almost 100% of both sexes of two other more sensitive strains (Fischer and Wistar) tested in the same experiment developed liver cancers (Table 5.14).

Benzidine dihydrochloride, administered to mice continuously in the diet at doses of 50, 100, and 150 ppm from the 6th to the 90th week of age, induced liver tumors at dose-related incidences of 6% to 44% in males and 26% to 94% in females (Vesselinovitch, Rao, and Milhailovich, 1975). When the carcinogen was administered intermittently instead of continuously, the sex difference was abolished (Table 5.15). Regardless of the schedule of administration, males developed more tumors of the Harderian gland than females. Lung adenomas were found in more males (23%) than females (7%), but only when feeding was intermittent. When the animals were fed the carcinogen twice (at 7 and then at 27 days of age), 66% of the males and none of the females developed liver tumors, whereas feeding at 42 and 62 days produced liver tumors in only 4% of the males and, again, none in the females. These data demonstrate the influence of age on tumor induction in males in particular.

Mulay and O'Gara (1959) demonstrated that Osborne-Mendel male rats fed *p*-dimethylamino-benzene-1-azo-1-naphthalene (DAN) and *p*-dimethylaminoazobenzene (DAB) were more sensitive to liver tumor induction than females. (See Table 5.14.) Daily administration of DAN induced liver tumors in 75% of the males and in 17% of the females, whereas DAB induced liver tumors in 85% of the males and 19% of the females. Male and female mice fed *p*-dimethylaminoazobenzene-1-azo-2-naphthalene (DA-2-N) responded with the same incidence of liver tumors (98% and 100%), but the tumors appeared more slowly in females, suggesting that they were more resistant to DA-2-N than were males. Baba and Takayama (1961), on the other hand, were unable to demonstrate sex differences in Wistar rats injected subcutaneously with DAB. The investigators suggested that if the experiment had been stopped on the 200th day, instead of on day 380, hepatoma incidence would have differed greatly between the two sexes, as induction time was longer for females. They also speculated that if the doses of carcinogen had been larger the difference in sex might have been obscured. The authors concluded that in DAB carcinogenesis sex of the animal does not affect the transformation of normal

Table 5.15. Comparison of continuous versus intermittent administration of BZ•2HCl on development of liver, Harderian gland, and lung

Dose (ppm)	Sex	Liver tumors				Harderian gland tumors				Lung adenomas			
		Continuous[a]		Intermittent[b]		Continuous		Intermittent		Continuous		Intermittent	
		Ratio[c]	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio	%
50	M	3/50	6	3/75	4	9/50	18	8/75	11	2/50	4	17/75	23
	F	13/50	26	4/75	5	3/50	6	2/75	3	2/50	4	5/75	7
100	M	11/50	22	12/75	16	18/50	36	11/75	15	3/50	6	19/75	25
	F	32/50	64	17/75	23	3/50	6	5/75	7	2/50	4	4/75	5

[a] Animals were given food containing specified amounts of BZ•2HCl.

[b] Mice received BZ•2HCl stomach intubation in amounts equivalent to specified dosages; mice were intubated twice weekly. The amounts at each treatment were 0.5 mg (corresponding to 50 ppm series) or 1.0 mg (corresponding to 100 ppm series) per animal at treatment.

[c] Number of mice bearing specified tumors per number of BZ•2HCl-treated animals.

Source: Adapted from Vesselinovitch, Rao, and Mihailovitch, 1975. Reprinted with permission of the publisher.

cells to malignant ones, but that it does affect proliferation of the cells and thus appearance of the cancer. This supports the opinion of Glucksmann and Cherry (1971) that sex-related differences in tumor induction are quantitative rather than qualitative.

Morris and Ferminger (1956), using 2-acetylaminofluorene, induced tumors in male and female rats (castrated and normal) that, in some cases, were treated with sex hormones. Most hepatomas were confined to the livers of intact males and of castrated females treated with testosterone propionate. Intact females, castrated males, castrated females, and castrated males treated with diethylstilbesterol were less susceptible (Table 5.14). Therefore, it appeared that under those experimental conditions the presence of androgen was important for the development of hepatomas in the rat. Estrogen did not seem to have a protective effect against tumor development.

Weisburger (1977) tested several halogen compounds for carcinogenicity in B6C3F$_1$ mice and in Osborne-Mendel rats. Some of the sex differences seen after 78 weeks of dosing by gavage were:

1. Chloroform-treated male rats had an increased incidence of kidney tumors, and females developed more thyroid tumors.
2. 1,2-Dibromo-3-chloropropane caused many mammary carcinomas in female rats.
3. 1,2-Dichloroethane caused some increase in hepatocellular carcinomas and stomach tumors in male mice and induced increased numbers of mammary tumors in female rats.
4. Iodoform increased thyroid tumors in male rats and hepatocellular carcinomas in male mice.

Both sexes of mice have been found to be equally susceptible to the hepatocellular effects of carbon tetrachloride (Andervont, 1958; Weisburger, 1977), whereas female Buffalo strain rats have appeared to be more sensitive than males (Reuber and Glover, 1967).

5.2.8 Conclusions

The need for lifetime exposures in chronic toxicity and carcinogenicity tests limits the choice of primary test animals to relatively short-lived rodents in all but a few exceptional cases. Rats are the overwhelming choice of most investigators for chronic testing, with mice and dogs distant second and third choices, respectively.

In addition to the primary test animal, most authorities recommend the use of a second species in chronic toxicity and carcinogenicity tests to reveal a broader range of toxic effects. Most, but not all, authorities recommend a nonrodent species for chronic tests. Among nonrodents, the dog is most frequently used, but other species may be selected if their metabolic processes are thought to resemble those of man more closely.

Rats, mice, and hamsters are generally selected for carcinogenicity assays. No test animal has been found to be an ideal surrogate for man in testing for long-term toxicity; however many species do respond to a variety of toxic substances and are reliable subjects for testing. Selection of the most appropriate species or strain for testing is highly complex and can depend on sensitivity desired by the experimenter, physiology, anatomy, and life span of the animal. Ideally, the test animal that most closely resembles man would be chosen, but cost and time requirements do not always allow this. The choice may be simplified through such techniques as those involving the use of structure-activity relationships, metabolism studies, and in vitro screening. These techniques, in conjunction with standardized animal studies, could aid in the efficient selection of the appropriate animal species. However, literature searches revealed a distinct lack of experiments expressly designed to demonstrate species differences in response to chemical carcinogens. Thus, it was necessary in this document to make many comparisons of nonstandardized data between experiments conducted in different laboratories. There is a definite need for basic, standardized animal experiments that allow for the comparison of the responses of various species to chemical carcinogens or classes of carcinogens.

The number of animals used in chronic toxicity and carcinogenicity tests represents a compromise between requirements for good statistical precision and reasonable costs and work loads. Early investigators used 10 or fewer test animals per dose group, but most authorities now recommend using 50 rodents or 4 to 8 nonrodents per group. Equal numbers of male and female animals are normally used.

Each long-term toxicity test should be evaluated with reference to a concurrent control group composed of animals of the same species, age, sex, and weight as the treated groups. These animals should be handled identically to the test species except for treatment with the test material. Each control group should contain at least as many animals as the corresponding test group. Negative and vehicle control groups are commonly used in all long-term tests, but positive controls are more frequently used in carcinogenicity testing.

General agreement exists among authorities on the need to start animals on test at an early age to provide maximum exposure during periods of active protein synthesis, cellular proliferation, and sexual maturation, as well as adulthood. In most routine screening tests for carcinogenesis, weanlings or immediately postweanling animals are used. There is also general agreement that newborn animals are sometimes even more sensitive to carcinogens than weanlings and adults; however, the metabolic, hormonal, and immunological incompetence of prenatal animals may induce responses uncharacteristic of more mature animals. Hence, some responses from newborn animals, taken alone, may not be reliable indicators of the carcinogenicity of the test material.

In principle, test sensitivity and age-related effects should be maximized if animals are exposed to the test material during all prenatal and postnatal phases of their lives. In practice, difficulties with dose estimation or regulation and teratogenic or reproductive interferences of the test material so limit the effectiveness of this approach that tests involving in utero and bigenerational exposures are performed only infrequently. As a consequence, much more experience with these methods will be required before their general application to carcinogenesis testing can be considered. However, use of such tests may be indicated whenever the chemistry or biology of the test compound, or its pattern of use, suggests a high level of interaction with in utero, infant, or preadolescent children.

The sex of the animal can influence the incidence, site, and latency of tumors. The host environment, which is determined by factors such as sex, appears to regulate the growth of the induced neoplasm. The mechanism of sex-influenced effects is not clear, and sex-related differences cannot be explained on the basis of hormone effects alone or on the basis of cell susceptibility. Homburger and Tregier (1960) described studies in rodents in which tumors induced in males grew larger when transplanted to males than when transplanted to females. These and results of previous tests indicated to them that cells of the male and female are equally susceptible to carcinogens but that the malignant cells encounter a less favorable environment in the female than in the male and that the tumors progress at a slower rate. Sex-related differences in the tumor response to chemical carcinogens can be altered by factors such as strain, species, and age of the animal; route and schedule of administration of the chemical; dose of the chemical; and duration of the experiment. Therefore, reliable testing of chemicals with unknown characteristics would be virtually impossible using only one sex of experimental animal.

5.3 ROUTES OF ADMINISTRATION

The mode of exposure of experimental animals to chemicals may influence the results of chronic and carcinogenicity studies and should closely simulate conditions under which human exposure would most likely occur (Ministry of Health and Welfare Canada, 1975). Thus, the oral route is preferred for testing compounds to which humans may be exposed through ingestion (Ministry of Health and Welfare Canada, 1975; World Health Organization, 1978). For gases, volatile industrial solvents, and other air contaminants, inhalation studies are recommmended (Clark, 1977; Nettesheim and Griesemer, 1978). Section 5.3.1 provides an evaluation of advantages and disadvantages of these exposure routes in tests for chronic toxicity and carcinogenicity and includes a description of other less common routes which have specific application in tests for carcinogenicity. In Sect. 5.3.2 examples are presented to illustrate differences or similarities observed in a given species following carcinogen exposure by different routes.

5.3.1 Exposure Routes

5.3.1.1 Oral Route - Suitable for all species, the oral route of exposure is used for testing compounds to which humans may be exposed through ingestion. Oral administration may be effected by gavage, by feeding in dietary mixtures, or by administration in drinking water. All three methods are useful for long-term administration and permit reliable quantitation if intake and body weight are measured routinely (Ministry of Health and Welfare Canada, 1975). Sontag, Page, and Saffiotti (1976) discussed the advantages and disadvantages of the three oral routes.

The gavage method has the advantages of more effective hazard control and better quantitation. The test agent can be freshly prepared and, because less test agent is needed, less storage space is required. Animal handling is increased, allowing frequent observation for clinical symptoms. Gavage has certain disadvantages: (1) intake of the test agent may be less than maximum, (2) a solvent is often required, (3) mortality may be increased because of trauma, and (4) animals must be closely matched by weight.

Feeding through diet mix and drinking water has other advantages and disadvantages. Although these methods most closely simulate the mode of human exposure and allow greater total intake, the quantity ingested may vary, homogenicity and stability of the mix may be inconsistent, and palatability may be altered. Furthermore, the concentration of the test material in the diet must be altered weekly or biweekly during the early part of the experiment to maintain a constant dose level (in milligrams per kilogram of body weight), because toxicity is related to dosage per unit of body weight and food consumption per unit of body weight decreases with increasing animal age (Weil, 1973). Also, potential exposure of workers who mix and dispense feed and water containing toxic chemicals must not be overlooked.

Dietary feeding is generally recommended for adult animals (Weisburger and Weisburger, 1967), and intubation techniques are reserved for newborn and infant animals and for chemically unstable compounds (Weisburger and Weisburger, 1967). Pills and capsules may be suitable for oral administration to large species. The Ministry of Health and Welfare Canada (1975) suggested experimental use of the oral route of exposure, in certain instances, even when it is not the primary mode of human exposure. However, substitution of the oral route would be feasible only if levels of the compound in the tissue are higher than those produced following administration by the normal route for humans. In any case, extrapolation from one route of exposure to another must be approached with extreme caution.

5.3.1.2 Repiratory Routes - Experimental exposure of the respiratory tract to chemical carcinogens is usually accomplished through inhalation or by intratracheal instillation. These and two other specialized techniques which are occasionally used for carcinogenicity studies (pellet implantation and tracheal washing) will be discussed. The most significant advantage of inhalation exposure is that it duplicates conditions under which humans are exposed to air contaminants and it is the most probable route of industrial exposure to toxic chemicals (Nettesheim and Griesemer, 1978; Ministry of Health and Welfare Canada, 1975). The disadvantages of inhalation techniques, some of which add difficulty to interpretation of results, were listed by Ministry of Health and Welfare Canada (1975): (1) methods and equipment are complex and expensive, (2) particle sizes must be exact, not to exceed 5 μ, (3) the respiratory anatomy of man and animals differs in several respects, and (4) it is difficult to determine the actual dose inhaled.

Of the various species of experimental animals, the rat seems to be most suited to inhalation studies. Size of the animal, economic considerations, and similarities to man (demonstrated by the induction of squamous cell carcinomas of bronchogenic origin) are desirable characteristics of the rat model (Page, 1977*b*).

To circumvent the complexity of inhalation procedures, other useful methods for direct exposure of the respiratory tract have been developed. Intratracheal instillation of test material permits administration of a controlled dose and can be used in cocarcinogenesis studies concomitantly with inhalation exposure (National Academy of Sciences, 1975). In addition, large particles and large doses can be administered, the nasal filter in rodents is bypassed, skin contamination is avoided, and there is less risk to personnel from carcinogen exposure. Intratracheal instillation is currently the method of choice for testing suspected respiratory carcinogens (Nettesheim and Griesemer, 1978). Disadvantages of the technique are site-to-site variability in deposition of the test material and the formation of foci of chronic inflammation in the respiratory tract. Hamsters appear to be the animal most used in experiments requiring intratracheal injections. Size of the animals, their low incidence of spontaneous respiratory infections, their low incidence of spontaneous tumors, and their susceptibility to various carcinogens make hamsters suitable for intratracheal instillation.

Implantation of carcinogen-containing pellets into respiratory tissues of the rat produced squamous cell carcinomas in the bronchial epithelium (Kuschner et al., 1957) and in the epithelium of heterotropic tracheal transplants (Griesemer et al., 1977). In the pellet implant system the carcinogen acts on a specific limited area determined by the investigator, and dose-response relationships have been established.

The respective carcinogenicities of two occupational pollutants, chromium compounds and polyurethane, have been detected using the bronchial implant. However, the acute and chronic traumas afforded by this method are unavoidable and can be troublesome.

Schreiber, Schreiber, and Martin (1975) described the induction of squamous cell carcinomas in the hamster using repeated localized tracheal washings with a carcinogen. Tumors produced by this method are easily detected (the animal shows respiratory distress as the tumor becomes large) and may usually be diagnosed simply by dissecting the trachea under the dissecting microscope (Nettesheim and Griesemer, 1978). This relatively new exposure technique is complex and traumatic to the animal, and little is known to date of its real potential.

5.3.1.3 Dermal Route — Of particular interest in carcinogenicity studies, this route exposes the skin directly and may result in exposure of the whole body following percutaneous absorption (Ministry of Health and Welfare Canada, 1975).

An obvious advantage of dermal application is the ease with which the latency for cancerous or precancerous lesions can be established (Page, 1977*b*). Also, the effective cutaneous dose can be smaller than the effective oral dose, even when absorbed (however, the maximum dose that can be given dermally is usually less than the maximum dose that can be administered orally); tumors arise rapidly, and they are usually multiple; the statistical analysis in skin carcinogenesis studies is advanced; the method is well suited to the two-stage carcinogenesis procedure; and the technique is useful for testing carcinogens that are destroyed in the gut. As for disadvantages, the procedure is arduous and expensive, the absorbed dose is not accurately known (whole body exposure may occur when the test agent is licked off the skin), lesions and infections caused by the vehicle may shorten the life span of the animal, and it is unsuitable for chemicals requiring activation in the gut (Ministry of Health and Welfare Canada, 1975).

Weisburger (1976) suggested that mice and rabbits are generally more responsive to dermal tumorigenesis than are other species. Magee (1970), on the other hand, concluded that there seemed to be no *inherent* difference in the capacity of mouse and rat skin to undergo malignant change. This conclusion was based partly on the results of Graffi, Hoffman, and Schutt (1967), who induced skin tumors in mice, rats, and hamsters by dermal application of *N*-nitrosomethylurea. However, Fare (1966) demonstrated that complete reliance on results of mouse skin carcinogenesis studies could be misleading. An azo dye, 3-methoxy-4-dimethylaminoazobenzene, when painted on the skin of ten male rats for 62 weeks, was a potent carcinogen, producing tumors in 100% of the animals. But the carcinogen was found to be totally ineffectual when painted on the skin of male and female mice for averages of 62 and 30 weeks, respectively.

5.3.1.4 Subcutaneous Injection — Subcutaneous injection is a sensitive and frequently used mode of administration of chemical carcinogens (Page, 1977*b*). Doses can be accurately delivered, absorption is slow, high concentrations in the blood are reached rapidly, and the compound circulates without those changes that might occur in the gut following oral administration. Repeated injections can be time-consuming and local reactions are likely to occur (Ministry of Health and Welfare Canada, 1975), but the appearance of subcutaneous sarcomas at the site of injection constitutes the most controversial characteristic of the technique. The production of subcutaneous sarcomas has been an end point in the testing of many compounds for carcinogenic potential. In 1941 Turner induced sarcomas in rats at the site of implantation of bakelite. Oppenheimer, Oppenheimer, and Stout (1948) obtained similar results using cellophane.

Grasso (1976), following extensive experiments performed in an attempt to elucidate the mechanism of induction of subcutaneous tumors at the site of injection or implantation, concluded that "a progressive type of connective tissue reaction, which has been carefully characterized histologically, is responsible for the malignant tumors that are produced in the subcutaneous tissue of rats and mice by the following: (*a*) solid implants, (*b*) injection of hypertonic, acidic, or surface active solutions, (*c*) injection of macromolecular substances or substances that form local deposits." Thus, although the subcutaneous techniques are still quite useful, caution is required in the interpretation of the results of assays in which they are used.

Homburger and Tregier (1960) observed species differences in susceptibility of hamsters, mice, rats, and monkeys to subcutaneously administered 3,4,9,10-dibenzopyrene. One monkey appeared to be resistant, but sarcomas were induced in hamsters, mice, and rats. Also, strain differences were observed among the mice tested.

5.3.1.5 *Other Exposure Techniques* - Intraperitoneal and intravenous injections and bladder implantation of wax pellets are less frequently used techniques.

The intraperitoneal route of administration is convenient for single-dose exposure to a carcinogen, the liver being the first organ encountered. With procarcinogens the tumor spectrum induced is the same as by oral administration (Weisburger, 1976), and injection may be done repeatedly if the carcinogen is soluble and if there is no vehicle accumulation. Intraperitoneal injection does not resemble the usual routes of human exposure.

Intravenous injection is a rapid and efficient means of delivery of test materials to various organs. However, it is an unlikely route for human exposure and is not well suited for repeated injections (Ministry of Health and Welfare Canada, 1975).

Jull (1951) introduced the technique of surgical implantation of wax pellets containing carcinogens into the bladders of mice. Since then other investigators, including Bonser et al. (1952) and Allen et al. (1957), have used the technique successfully for testing aromatic amines, polycyclic hydrocarbons, saccharin, and other potential carcinogens. However, the U.S. Food and Drug Administration (1971) felt that the number of chemicals tested comparatively by both bladder implantation and more conventional methods was insufficient to provide sound interpretation of results obtained by the technique. Therefore, any chemical clearly carcinogenic by bladder implantation would be considered suspicious but should then be tested by more conventional assays.

5.3.2 Carcinogen Exposure by Different Routes

5.3.2.1 Diethylnitrosamine in the Hamster — The effects of route of administration on the carcinogenic action of diethylnitrosamine have been tested in the Syrian hamster (Herrold and Dunham, 1963; Herrold, 1964*a*, 1964*b*). The routes tested were intragastric, intratracheal, subcutaneous, intradermal, topical, and intraperitoneal. Results of the experiment are summarized in Table 5.16. Irrespective of the route of administration of diethylnitrosamine, tumors were induced in the trachea, bronchi, nasal cavity, and liver of the hamsters. The frequency of tumors in the nasal cavity was considerably higher after subcutaneous administration of diethylnitrosamine than after administration by intragastric or intratracheal routes (Herrold, 1964*a*). Although liver tumors did not develop in animals injected intratracheally, atypism of the hepatic cells was observed (Herrold and Dunham, 1963). There was no local effect on the skin or in subcutaneous tissue (Herrold, 1964*b*). Transplacental transfer of the carcinogen was not demonstrated by Herrold, but Mohr (1973) described extensive experiments in which he and his associates induced tumors transplacentally with diethylnitrosamine.

Table 5.16. Induction of tumors in the Syrian hamster with diethylnitrosamine

Route	Number of animals	Dose	Exposure schedule	Tumor incidence						Reference
				Trachea	Bronchi	Liver	Nasal cavity		Kidney[d]	
							Anterior	Posterior		
Buccal pouch	10	0.1 mg in saline (total = 7.5 mg)	Twice a week for about 9 months	0/10	0/10	0/10	0/10	0/10		Millevskaja and Kiseleva, 1976
Intradermal	19	3.5 mg in water (total = 70–84 mg)	Once a week for 5–6 months	19/19	10/19	3/19	10/19	13/19		Herrold, 1964a
Intraperitoneal	18	2 mg in saline (total = 32–56 mg)	Once a week for 4–7 months	17/18	4/18	4/18	5/15	11/15		Herrold, 1964a
Topical	8	Undiluted,[b] then 1:1 in water	Once a week for 1 month then twice a month for 3 months	6/8	2/8	4/8	6/8	4/8		Herrold, 1964a
Pregnant female subcutaneous	3	5, 8, or 10 mg in water	1–2 days preceding delivery (single dose)	3/3	1/3	0/3	1/3	2/3		Herrold, 1964a
Subcutaneous	15	2 mg in water (total = 32–48 mg)	Twice a week for 4–6 months	NS[c]	NS	NS	14/15			Herrold, 1964a
Intratracheal	11 female	0.05 mL of a 1:14 solution in water[d]	Once a week for 6 months	11/11	4/11	0/11	3/9		0/11	Herrold and Dunham, 1963
	14 male			14/14	10/14	0/14	2/13		0/14	
Intragastric	15 female	0.4 mL of a 1:250 solution in water	Twice a week for 6 months	15/15	6/15	10/15	6/11		7/15	Herrold and Dunham, 1963
	13 male			13/13	4/13	12/13	4/9		5/13	

[a] Microscopic lesions that may represent an early stage in tumorigenesis.

[b] Dose and schedule were changed after animals died during the first month of treatment.

[c] NS – tumors observed but incidence not specified.

[d] Doses administered intratracheally and intragastrically were approximately the same.

Milievskaja and Kiseleva (1976) attempted to induce tumors in the buccal pouch of the Syrian hamster with injections of diethylnitrosamine. Doses of 0.1 mg per animal twice a week for about 9 months failed to produce local or remote tumors. The small number of animals (ten) or the route of administration constitutes possible reasons for the lack of carcinogenicity of diethylnitrosamine in the experiment. The total dose administered to the buccal pouch, however, was only one-tenth of the intradermal dose required to produce tumors in hamsters (Herrold, 1964b); therefore, the lack of effect could be attributed to insufficient dose.

5.3.2.2 Methylcholanthrene in the Mouse — Skin carcinomas have been induced in mice painted with single large doses (0.1 mL of 0.6% solution four times in an hour) or with repeated smaller doses (0.15% two times per week for 30 weeks) of 3-methylcholanthrene (Bielschowsky and Bellough, 1949; Shubik, 1950). Table 5.17 contains experimental details for local tumor induction with methylchloanthrene.

When methylcholanthrene is painted on the skin of mice in doses too low to produce tumors, croton oil, applied repeatedly, enhances the appearance of tumors (Klein, 1952). The promoting action of croton oil in skin carcinogenesis appeared to be a factor in the induction of sarcomas in albino mice following intramuscular injection of methylcholanthrene (Klein, 1951). Single injections of 0.024 or 0.004 mg of methylcholanthrene in croton oil produced tumors in a shorter time and with a higher incidence than those produced by the same doses of carcinogen injected alone. However, croton oil did not influence tumor number or latency when injected intramuscularly with 0.002, 0.0008, or 0.0004 mg methylcholanthrene doses, which produced few or no sarcomas at the site of injection (Klein, 1953).

Subcutaneous administration of polycyclic hydrocarbons has been used primarily for the induction of connective tissue tumors. Epidermal changes that occur with this route of exposure are not often described. Bhisey and Sirsat (1975) monitored histological changes in the skin of Swiss albino mice at various times after subcutaneous injection of 0.5 mg of 20-methylcholanthrene in thiophene-free benzene. A mild focal epidermal hyperplasia developed, which led to development of squamous

Table 9.17. Local tumor induction in mice with 3-methylcholanthrene by various routes of administration

Route	Strain	Number of animals	Methylcholanthrene dose	Exposure schedule	Average induction time	Tumor incidence	Reference	Comments
Skin painting	Kreyberg	30	0.1 ml of 0.6% in acetone	4 applications at 15-min intervals	10.5 weeks	7/30 (skin)	Bielschowsky and Bullough, 1949	3/7 carcinomas (partial autopsy)
	CF-1	28	0.15% in acetone	Twice a week for 30 weeks	Not clear	23/28 (skin)	Shubik, 1950	2 malignancies in 69 total tumors (partial autopsy)
	DBA	34	0.5% in olive oil	Once, followed by application of 5% croton oil in olive oil 1-3 times per week for 53 applications	59 days	27/34 (skin)	Klein, 1952	
		35	0.5% in olive oil	Once, with no additional treatment		0/35 (skin)	Klein, 1952	
Intramuscular	Albino A	40	0.024 mg in olive oil	Single injection	160 days	20/40 (muscle)	Klein, 1951	Fibrosarcomas (partial autopsy)
		40	0.024 mg in olive oil and croton oil	Single injection	95 days	40/40 (muscle)	Klein, 1951	Fibrosarcomas (partial autopsy)
		32	0.004 mg in olive oil	Single injection	168 days	9/32 (muscle)	Klein, 1951	Fibrosarcomas (partial autopsy)
		32	0.004 mg in olive oil and croton oil	Single injection	123 days	22/32 (muscle)	Klein, 1951	Fibrosarcomas (partial autopsy)
		35	0.002 mg in olive oil	Single injection	183 days	5/35 (muscle)	Klein, 1953	Fibrosarcomas (partial autopsy)
		36	0.002 mg in olive oil and croton oil	Single injection	187 days	3/36 (muscle)	Klein, 1953	Fibrosarcomas (partial autopsy)
		36	0.0008 mg in olive oil	Single injection	254 days	1/36 (muscle)	Klein, 1953	Fibrosarcomas (partial autopsy)
		35	0.0008 mg in olive oil and croton oil	Single injection	272 days	1/35 (muscle)	Klein, 1953	Fibrosarcomas (partial autopsy)
		33	0.0004 mg in olive oil	Single injection		0/33 (muscle)	Klein, 1953	
		37	0.0004 mg in olive oil and croton oil	Single injection		0/37 (muscle)	Klein, 1953	

Table 5.17 (continued)

Route	Strain	Number of animals	Methylcholanthrene dose	Exposure schedule	Average induction time	Tumor incidence[a]	Reference	Comments
Subcutaneous	Swiss	24	0.3 mg in thiophene-free benzene	Single injection		13/24 (skin) Sirsat, 1975	Bhisey and Sirsat, 1975	Squamous cell carcinoma (partial autopsy)
						2/24 (muscle)		Rhabdomyosarcomas (partial autopsy)
						19/24 (subcutaneous connective tissue)		Fibrosarcomas (partial autopsy)
						1/24 (skin)		Papillomas (partial autopsy)
						4/24 (skin)		Sebaceous cysts (partial autopsy)
Intratracheal	B6C3F$_1$	36	0.3 mg in 0.2% gelatin	Once a week for 6 weeks	(First mouse died in 4 weeks, last in 24 weeks)	11/36 (lung)	Nettesheim and Hammons, 1971	Squamous cell carcinomas
	DBA/2	30	0.3 mg in 0.2% gelatin	Once a week for [illegible] weeks		1/18 (lung)	Nettesheim and Hammons, 1971	Squamous cell carcinomas (22 animals were still alive)
Bladder implant of pellets	Albino	37	12.5% suspension	Continuous up to [illegible] weeks		18/37 (urinary bladder)	Bonser et al., 1963	Carcinomas (partial autopsy), 12% of controls developed tumors
		38	20% suspension	Continuous up to [illegible] weeks		22/38 (urinary bladder)	Bonser et al., 1963	Carcinomas (partial autopsy), 4.8% of controls developed tumors

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[a] Number of tumor-bearing mice per total number of mice at risk.

cell carcinomas. Papillomas, sebaceous cysts, rhabdomyosarcomas, and fibrosarcomas also developed. Bhisey compared the overall lack of epidermal hyperplasia seen in these experiments to the massive epidermal hyperplasia thought to be a required step during neoplastic transformation of mouse skin topically painted with methylcholanthrene. He concluded that "when methylcholanthrene is administered subcutaneously to Swiss mice, epidermal hyperplasia is not a prerequisite for epidermal tumorigenesis."

Examples of specialized techniques for local tumor induction in the respiratory tract and urinary bladder include intratracheal injection and pellet implantation, respectively. Repeated intratracheal injections of 3-methylcholanthrene have induced squamous cell carcinomas in the respiratory tracts of mice (Nettesheim and Hammons, 1971). When DBA/2 and $BC3F_1$ mice were injected weekly with 0.5 mg of 3-methylcholanthrene for 4 and 6 weeks, respectively, 31 of the 36 $BC3F_1$ mice developed squamous cell carcinomas, the first appearing 4 weeks after exposure. Tumor incidence was much lower in the DBA/2 strain. Methylcholanthrene-impregnated (12% or 20% methylcholanthrene) wax pellets implanted in the urinary bladder of albino mice induced local squamous cell carcinomas in approximately 55% of the animals during 25 weeks of observation (partial autopsy) (Bonser et al., 1963).

Systemic effects of methylcholanthrene in mice have been reported following oral, intravenous, subcutaneous, and vaginal exposure. Akamatsu and Barton (1974) fed, by gavage, 1 mg of 3-methylcholanthrene in olive oil to five inbred strains of mice. Tumors were induced at various sites, including skin, forestomach, liver, lung, and lymphatics (Table 5.13). Treated animals also developed amyloidosis, which was significantly correlated with gastric neoplasms.

Strain A mice, highly susceptible to chemical induction of pulmonary adenomas, developed an increased number (as compared with controls) of lung tumors 4 weeks after intravenous injection of 0.05 to 1.5 mg methylcholanthrene in horse serum and cholesterol (Table 5.18) (Shimkin, 1940). The incidence of tumors was 100% in 3 months. Subcutaneous injection of 0.25, 0.5, and 1.0 mg of methylcholanthrene in lard induced lung tumors in strain A mice, with 70%, 80%, and 91% incidence developing

Table 5.18. Incidence of pulmonary tumors in strain A mice after intravenous injection of methylcholanthrene

Time (weeks)	Dosage								
	1.5 mg			0.5 mg			0.1 mg		
	Number of mice	Number with tumors of the lungs	Average number of tumors of the lungs	Number of mice	Number with tumors of the lungs	Average number of tumors of the lungs	Number of mice	Number with tumors of the lungs	Average number of tumors of the lungs
[illegible]	6	1	1	9	1	1			
[illegible]	7	7	12	10	8	5			
5	10	10	22	10	10	14			
6	4	4	55	10	10	25	7	3	2
13	5	5	74	18	18	30	10	8	4
20				6	6	47	15	15	11

Source: Adapted from Shimkin, 1940. Copyright 1940, American Medical Association. Reprinted with permission of the publisher.

by 3 months. Differences seen in carcinogenic potency of 20-methylcholanthrene (in horse serum) and 1,2,5,6-dibenzanthracene (in cholesterol), when 0.5 mg of each compound was injected intravenously, disappeared when 0.5 mg of each was injected subcutaneously (Tables 5.19 and 5.20). These results suggest that the subcutaneous route is less sensitive than the intravenous route for lung tumor induction in strain A mice.

Table 5.19. Incidence of pulmonary tumors in strain A mice after intravenous injection of 0.5 mg of methylcholanthrene or 0.5 mg of dibenzanthracene

	Methylcholanthrene			Dibenzanthracene		
Time (weeks)	Number of mice	Number with tumors of the lungs	Average number of tumors of the lungs	Number of mice	Number with tumors of the lungs	Average number of tumors of the lungs
3	9	1	1	10	0	0
4	10	8	5	10	3	1
5	10	10	14	11	10	3
6	10	10	25	10	10	8

Source: Adapted from Shimkin, 1940. Copyright 1940, American Medical Association. Reprinted with permission of the publisher.

Table 5.20. Incidence of pulmonary tumors in strain A mice given subcutaneously 0.5 mg of methylcholanthrene or 0.5 mg of dibenzanthracene dispersed in 0.5 cc of horse serum and cholesterol

	Methylcholanthrene			Dibenzanthracene		
Time (weeks)	Number of mice	Number with tumors of the lungs	Average number of pulmonary tumors	Number of mice	Number with tumors of the lungs	Average number of pulmonary tumors
3	10	0	0	10	0	0
4	10	2	1.0	7	0	0
5	10	5	1.6	6	2	1.0
6	10	7	2.2	7	5	2.0

Source: Adapted from Shimkin, 1940. Copyright 1940, American Medical Association. Reprinted with permission of the publisher.

Intravaginal exposure of ICR Swiss mice to 20-methylcholanthrene gave rise to tumors remote from the site of application (Campbell, Yang, and Bolton, 1965). During a 6-week period, doses totaling 1.5 to 5.5 mg of 20-methylcholanthrene were administered, and the mice were observed for life. Of 106 female mice, 23% developed lung adenomas (two mice had adenocarcinomas), 12% developed genital tract cancer, and genital tract dysplasia occurred in 27%. Six of the 24 mice with lung adenomas also had cancer and/or dysplasia of the vagina and/or cervix uteri.

5.3.2.3 Aromatic Amines in Mice, Rats, and Rabbits – Bonser et al. (1952) compared carcinogenesis of 2-naphthylamine and 2-amino-1-naphthol hydrochloride in several species by several routes of administration. Implantation of 2-amino-1-naphthol pellets (10% to 15% carcinogen) induced metaplasia, papilloma, and carcinoma in the bladders of mice, and subcutaneous injection (5 mg per 100 g of body weight) induced sarcomas in both mice and rats. Implantation of 2-naphthylamine pellets in the bladder and skin painting with the carcinogen failed to produce bladder tumors in mice, but oral administration (100 to 300 mg/kg weekly) induced hepatomas in mice and benign bladder tumors in rats and rabbits (Table 5.21).

Inhalation of benzidine causes bladder cancer in man (Goldwater, Rosso, and Kleinfeld, 1965) and has been shown to be carcinogenic in certain animal species. Rats, after inhaling benzidine for 13 months, developed leukemia, fibroadenomas, carcinoma of the mammary glands, carcinoma of the male mammary gland, and hepatoma, but no bladder tumors (Zabezhinskii, 1970). Oral administration of benzidine to rats induced hepatomas and rectal and acoustic duct carcinomas (Spitz, Maguigan, and Dobriner, 1950), male mammary gland carcinomas, and leukemia (see National Institutes of Health, 1968-1973). The absence of tumors of the urinary bladder of rats receiving benzidine by inhalation and by subcutaneous and oral administration led Zabezhinskii to conclude that the character of action of aromatic amines is dependent mainly on the species of the experimental animal and not the mode of administration of the substance.

Table 5.21. Tumor induction with aromatic amines by various routes of exposure

Chemical/species	Route of exposure	Dose	Duration of exposure (weeks)	Tumor incidence	Tumor latency (weeks)	Reference
2-Amino-1-naphthol hydrochloride						
Mouse	Bladder pellet	1–2 mg per pellet	22 to 28 30 to 39	0/6 bladder tumor 1/8 bladder adenoma 5/8 bladder carcinoma	About 30	Bonser et al., 1952
Mouse, "stock"	Subcutaneous	5 mg per 100 g body weight every 2 weeks	Up to 80	2/15 subcutaneous tumors 3/15 leukemias 4/15 hepatomas		Bonser et al., 1952
Mouse, R III	Subcutaneous	5 mg per 100 g body weight every 2 weeks	Up to 80	1/8 subcutaneous tumors 1/8 leukemia (hepatoma and leukemia in controls)		Bonser et al., 1952
Rat, albino	Subcutaneous	5 mg per 100 g body weight every 2 weeks	Up to 80	5/14 subcutaneous tumors		Bonser et al., 1952
2-Naphthylamine						
Mouse	Bladder pellets	1–2 mg per pellet	39	0/8 bladder tumor		Bonser et al., 1952
Mouse, IF	Gavage	5 mg every 2 weeks in arachis oil	Up to 72	Cholangioma of bile duct		Bonser et al., 1952
Mouse, CBA	Gavage	120 mg/kg body weight twice per week	Up to 89	13/23 hepatomas		Bonser et al., 1952
	Diet	160 mg/kg body weight per week	Continuous	11/26 hepatomas	About 60	Bonser et al., 1952
Rat, albino	Diet (low protein)	310 mg/kg body weight per week	90	3/15 bladder papillomas	About 60	Bonser et al., 1952
Mouse, IF	Skin painting	Saturated solution	Up to 99	0/25 skin tumor 0/25 hepatoma		Bonser et al., 1952
Rabbit	Spoon feeding	100 mg/kg body weight per week	Up to about 275	1/6 bladder papilloma		Bonser et al., 1952

Table [illegible] (continued)

Chemical/species	Route of exposure	Dose	Duration of exposure (weeks)	Tumor incidence	Tumor latency (weeks)	Reference
Benzidine						
Rat, noninbred	Inhalation	4 hours/day, 5 days/week	80	3/28 leukemias 1/28 hepatoma 2/28 mammary tumors	52	Zabizhinskii, 1970
Benzidine, technical						
Rat	Subcutaneous	15 mg/week	Life	8/233 hepatomas 0/233 bladder tumors 7/385 gastric tumors 54/233 auditory canal tumors		Spitz, Maguigan, and Dobriner, 1950
Benzidine, pure						
Rat	Subcutaneous	15 mg/week	Life	6/152 hepatomas 0/152 bladder tumors 32/152 auditory canal tumors		Spitz, Maguigan, and Dobriner, 1950
Benzidine sulfate						
Rat	Subcutaneous	15 mg/week	Life	1/153 hepatomas 0/153 bladder tumors 16/153 auditory canal tumors		Spitz, Maguigan, and Dobriner, 1950
	Oral	15 mg/week	Life	1/37 hepatomas 0/37 bladder tumors 2/37 auditory canal tumors		Spitz, Maguigan, and Dobriner, 1950

5.3.3 Conclusions

It is recommended that the route of exposure used in animal studies closely resemble the means by which man would encounter the chemical in the environment. Nevertheless, it is sometimes necessary to substitute other routes. Because of the variables inherent in any animal test system – metabolic, biochemical, species, and strain differences, for example – route-to-route extrapolations should be made with extreme caution. Experiments designed specifically to define the role of route of administration of chemicals to animals in carcinogenesis studies are not easily found in the literature. However, it is obvious that route of administration is a major modifying factor that can determine site, histological type, and incidence of tumors.

5.4 DOSE AND DURATION

Dose selection is a critical aspect of chronic toxicity and carcinogenicity testing. If the dose level is set too low, no effects will be observed; if it is set too high, excess mortality rates will reduce the test population too low for adequate statistical analysis. Consequently, dose rates for long-term testing should be set only after data from acute and subchronic tests have been analyzed. When possible, pharmacokinetic data should also be considered (Golberg, 1975; Munro, 1977). Massive doses that result in nonlinear pharmacokinetics should be avoided unless such data reflect actual conditions of human exposure (Watanabe, Young, and Gehring, 1977).

In early chronic toxicity testing, the primary purpose was the demonstration of no-observed-effect level; more recently, data needed for dose-response analysis have also been sought (Barnes and Denz, 1954; *Federal Register*, 1979; National Academy of Sciences, 1977; Winstead, 1978). For the establishment of a no-observed-effect level, at least three dose rates are required. The highest level should produce some signs of toxicity without seriously altering normal physiological function or causing excessive lethality during the course of the experiment. The lowest level should be a fraction of the high dose that is not expected to produce evidence of toxicity. The remaining dose should be set at a level intermediate between the high and low dose (World Health Organization, 1978). Dose levels should also be influenced by the anticipated level of human exposure as well as the margin of safety that may be desired in setting subsequent exposure standards (National Academy of Sciences, 1977). If the determination of a dose-response curve is an objective of the chronic toxicity test, more than three dose levels may be required (*Federal Register*, 1979; Food Safety Council, 1978).

Although a test for carcinogenic potential might occasionally entail only a single well-selected dose, tests for risk estimation or determination of a no-effect level usually require several dose levels (Page, 1977a). If more than one dose is used, the highest level should be within the toxic range but should be consistent with prolonged survival of the majority of the animals. The lowest dose selected ideally should produce no real increase in tumor incidence over controls (Ministry of Health and Welfare Canada, 1975). The recommendations of several agencies regarding the controversial subject of dose selection in carcinogenicity studies are presented in Table 5.22.

Weisburger (1976) emphasized the selection of the maximum tolerated dose (dose at which mortality is low in an 8-week test and which may depress weight gain 5% to 20%, with an optimal depression of approximately 10%). The rationale given by Munro (1977) for use of maximum tolerated dose (MTD) includes: (1) when weak carcinogens are tested, high doses for long periods of time increase chances of seeing statistically increased numbers of tumors within the life span of the animals (since cancer induction in animals is both time- and dose-related); and (2) increased confidence can be placed in negative data because the chemical has been tested at the maximum dose level compatible with normal or near normal survival rates. Munro also points out several problems associated with the use of MTD: (1) the fact that MTD may result in a 10% weight loss is considered to be inappropriate because it may affect biochemical or physiological processes that make the animal more or less susceptible to carcinogenic challenge; (2) metabolic overloading may result in changes in normal handling of the test compound; (3) simultaneously induced toxicity due to the test compound may enhance or diminish its carcinogenic activity, and further use of MTD favors

Table 5.22. Summary of recommendations on dose selection

Agency	Recommendation
Health Protection Branch, Department of National Health and Welfare Canada, 1973	"The highest dose level within the toxic range but consistent with prolonged survival. The lower levels should permit . . . good health . . . until tumors develop."
International Union Against Cancer, 1969	"All substances submitted for carcinogenicity testing should be examined at 3 or more dose levels."
National Cancer Institute, 1976	"The MTD[a] is defined as the highest dose of the test agent given during the chronic study that can be predicted not to alter the animals' normal longevity from effects other than carcinogenicity. The MTD should be the highest dose that causes no more than 10% weight decrement, as compared to the appropriate control groups and does not produce mortality, clinical signs of toxicity, or pathologic lesions (other than those that may be related to a neoplastic response) that would be predicted to shorten the animal's natural life span. Although depressed weight gain may be a clinical sign of toxicity, it is acceptable when estimating the MTD. Since the data may not always be easily interpretable, a degree of judgment is often necessary in estimating the MTD."
U.S. Food and Drug Administration, 1971	"At several dose levels — one likely to yield maximum tumor incidence."

[a] MTD — Maximum tolerated dose.

Source: Adapted from Munro, 1977. Reprinted with permission of the publisher.

very toxic compounds that can be used only at lower dose levels, thus reducing the chance of tumor formation; (4) there is difficulty in handling data when the criteria for MTD are not met; and (5) MTD doses usually far exceed human exposure levels.

In tests for carcinogenicity a dose-response relationship should be established if at all possible, but, because of the number of animals required, this is highly impractical with doses that produce lower tumor incidences (Terracini, Magee, and Barnes, 1967). Also, the limited life span of the animals may frustrate attempts to establish a low dose response. The simple concept of additive effects of repeated doses is not applicable to every situation. Page (1977*b*) suggested that the effectiveness of increasing exposure might decrease on a per dose basis for some chemicals especially if the body's ability to retain the chemical becomes saturated. This is illustrated by the study of Schepers (1971), who compared the effects of beryllium oxide in rats after: (1) continuous inhalation exposure up to 12 months, (2) continuous exposure for 6 months and (3) brief exposure for 1 month to high concentrations of the chemical. The rate of tumor formation was enhanced in animals of the interrupted (6-months) exposure group, whereas those exposed continuously for a long period and those exposed continuously for a shorter period (but to a higher concentration) developed tumors at about the same slow rate. Schepers postulated that the effect might be attributed to saturation of the lung with beryllium oxide.

Carcinogens are usually administered every day (to simulate human exposure) over the life span of the animal; some experiments may be terminated when mice are 18 months old and rats are 24 months old (Ministry of Health and Welfare Canada, 1975). Termination of studies must be carried out with caution since some tumors tend to develop late in the life of the animal. For example, Nelson, Fitzhugh, and Calvery (1943) reported that rats did not begin to develop liver tumors until 18 months after they had been placed on a diet containing selenium. The tumors in 11 of 53 animals arose in cirrhotic livers, while control animals had a spontaneous hepatic tumor incidence of less than 1%.

In carcinogenicity testing the route of administration often determines the frequency of exposure necessary for tumor development. Weisburger and Weisburger (1967) pointed out that for cutaneous exposure to strong carcinogens a weekly or even a single treatment is satisfactory. More frequent administration of weak carcinogens is recommended. Intraperitoneal injections require a repeated schedule because of the higher absorption rate of the internal organs, while intravenous administration allows no more than approximately eight injections.

In chronic toxicity evaluations the test compound should also be administered 7 days per week (Benitz, 1970). Treatment 5 days a week results in a 28.6% decrease of exposure and a 48-h weekly recovery period. The administration of the equivalent of seven doses during 5 days as recommended by some workers (Boyd, 1968) does not constitute a treatment equivalent to daily dosing and may not produce identical results (Baker and Alcock, 1965, as cited in Benitz, 1970; Hayes, 1967).

Apart from dose rate, length of exposure is the principal characteristic that distinguishes chronic tests from other forms of toxicity testing. Most investigators agree that chronic testing should extend over a large portion of the test animal's life span (Boyd, 1968; Food Safety Council, 1978; National Academy of Sciences, 1975, 1977; World Health Organization, 1978); however, there is no consensus concerning the length of this period. Conceptually, the duration of a chronic toxicity test should reflect the time for the test material, or its active metabolites, to accumulate to toxic levels in the organism, plus the time required for the target organ or system to respond once the toxic concentration is achieved (Lawrence, 1976). Generally speaking, most effects of chronic testing become apparent within 3 or 4 months. For example, in one study of the chronic effects of 11 drugs on rats, dogs, and monkeys, all compounds produced an observable effect within the first 2 weeks, and only one compound caused an additional effect after the first 3 months (Peck, 1968). Similarly, in a chronic study of 46 compounds, the Ciba Drug Company noted all indications of toxicity within the first 8 weeks of the test (Bein, 1963, as cited in McNamara, 1976). In this connection, McNamara (1976) stated, "If no effect occurs in 3 months, there is a low likelihood that any effect will occur on continued dosing for 1 year." Barnes and Denz (1954) asserted that little useful information can be extracted from chronic toxicity tests after the first 3 to 6 months. Because of this characteristically early detection of most chronic effects, many investigators feel that the duration of chronic tests need not exceed 6 months (Barnes and Denz, 1954; McCollister, 1974, as cited in McNamara, 1976; Paget, 1963, as cited in McNamara, 1976; Weil and McCollister, 1963; Zbinden, 1973). However, this conclusion appears to have gained wider acceptance among investigators dealing with drugs, to which humans may be exposed for relatively brief intervals, than with investigators dealing with occupational and environmental pollutants, to which the population may be exposed for intervals approximating a lifetime. For assessing the effects to humans of lifetime exposures of toxic compounds, some scientists insist that lifetime or near-lifetime exposures of laboratory test animals are required (Food Safety Council, 1978; Loomis, 1974; National Academy of Sciences, 1975; World Health Organization, 1978). Unlike the shorter acute or subchronic tests, lifetime exposures are said to reveal toxic effects associated with (1) repetitive exposures to a test material and (2) changes occurring during the aging process, such as altered tissue sensitivity, changing metabolic and physiological capability, and spontaneous disease (World Health Organization, 1978). The logic of the latter argument appears unassailable; however, examples illustrating its validity do not abound in the literature.

Only occasionally are chronic effects reported that were not observed during the first 90 days of treatment (Page, 1977b). Among more than 100 computer-selected publications on chronic toxicity examined during the preparation of this chapter (see Bibliography), only two such papers were noted: (1) Rosenkrantz et al. (1975) reported observing hyperglycemia in rats only after 180 days of treatment with Δ^9-tetrahydrocannabinol, and (2) Verschuuren, Kroes, and Van Esch (1973) detected stridor in rats fed tetrasul only after 4 months of treatment. In a similar examination of the literature, McNamara (1976) considered 82 long-term toxicity studies involving 122 test materials and 566 dose levels. Only three compounds and 15 dose levels produced toxic signs after, but not before, 3 months exposure. It thus appears that in most instances all significant toxic effects are indeed observed within 3 to 4 months, but that exceptions occasionally occur. Because it is not possible to determine a priori the relative significance of the occasional chronic effect missed in short-term tests, confidence in the validity of toxicity test results can be maximized only by performing life span exposures on test animals, even though longer tests are much more expensive and will provide no additional useful information most of the time. Justification of the test duration thus becomes a discretionary decision that must be resolved on the basis of risk-benefit analysis.

When lifetime or near-lifetime exposures of test animals are required in chronic toxicity tests, the actual duration of exposure will naturally vary with test animal. Many strains of mice or rats will survive 30 months or more with good care; consequently, these animals are usually exposed for 24 to 27 months, beginning at weaning (Food Safety Council, 1978; National Academy of Sciences, 1977). The normal life spans of other common test animals are considerably greater than that indicated for mice and rats. Table 5.23 shows that chronic tests of even 2 years duration represent only a small fraction of the life span of most nonrodent laboratory test animals. Needless to say, these animals are only rarely chosen for life span exposures. Among the publications examined during the preparation of this chapter, no life span exposures of nonrodents were found, but one 7-year dog study and one 43-month monkey study were noted.

When a long-lived animal is chosen as a second species to mice or rats in chronic toxicity tests, exposures amounting to at least 10% of the projected life span of the animal are generally recommended (National Academy of Sciences, 1977; World Health Organization, 1978). On this basis, dogs used as a second species in chronic toxicity tests should usually be exposed for at least 1 year. However, several investigators have reported previously unobserved toxic effects in dogs after periods of exposure greater than 1 year (Braun et al., 1977; Kaplan and Sherman, 1977; Weil et al., 1971). Consequently, some authorities recommend exposing dogs longer, for periods of 2 to 7 years (Goldenthal, 1968; Loomis, 1974; National Academy of Sciences, 1977). In the chronic toxicity papers analyzed in this study the typical duration of exposure of dogs used as a second species was 2 years.

In conclusion, dose selection is a critical aspect of long-term testing. Dose rates should be set only after acute and subchronic test data have been analyzed and pharmacokinetic effects have been considered. At least three dose rates are required to establish a no-observed-effect level. The highest level should produce some signs of toxicity without seriously altering normal physiological function or causing excessive lethality during the course of the experiment. The lowest level should be a fraction of the high dose that is not expected to produce evidence of toxicity. The remaining dose should be set at an intermediate level

Table 5.23. Time relationships among drug exposure, life span, and time equivalents in man

Duration of study (months)	Rat		Rabbit		Dog		Pig		Monkey	
	Life span (%)	human equivalent (months)	Life span (%)	Human equivalent (months)	Life span (%)	Human equivalent (months)	Life span (%)	Human equivalent (months)	Life span (%)	Human equivalent (months)
1	4.1	34	1.5	12	0.82	6.5	0.82	6.5	0.55	4.5
2	8.2	67	3.0	24	1.6	14	1.6	14	1.1	9
3	12	101	4.5	36	2.5	20	2.5	20	1.6	13
6	25	202	9.0	72	4.9	40	4.9	40	3.3	27
12	49	404	18	145	9.8	81	9.8	81	6.6	53
24	99	808	36	289	20	162	20	162	13	107

Source: Adapted from Benitz, 1970.

between the high and low doses. Dose levels should also reflect anticipated human exposures as well as the margin of safety that may be desired in setting subsequent exposure standards. If a dose-response relationship is a goal of the test, then more than three dose levels may be required.

The test compound should be administered 7 days per week; treatment 5 days a week or administration of the equivalent of seven doses during 5 days is not equivalent to daily dosing and may not produce identical results. In carcinogenicity studies, the exposure pattern is often modified by the route of exposure.

Most authorities agree that chronic toxicity and carcinogenicity studies should extend over a large portion of the test animal's life span. However, there is no consensus concerning the length of this period. Conceptually, the duration of a chronic toxicity or carcinogenicity test should reflect the time for the test material, or its metabolites, to reach toxic concentrations in the test animal, plus the time required for the target organ or system to respond once the toxic concentration is achieved. Generally speaking, most effects of noncarcinogenic chronic testing become apparent within 3 or 4 months, and many authorities feel there is only a low likelihood that additional effects will be observed after 6 months of treatment. Nevertheless, a few new effects are occasionally seen during longer periods of treatment, and in carcinogenicity studies the chance for late development of long-term effects is even greater. Because it is not possible to determine a priori the relative significance of the occasional chronic effect missed in short-term tests, confidence in the validity of toxicity test results can be maximized only by performing life span exposures on test animals, even though the longer tests are much more expensive and will provide no additional useful information most of the time.

When lifetime or near-lifetime exposures of test animals are required in chronic toxic and carcinogenicity tests, the actual duration of exposure naturally varies according to species. The life spans of mice and rats are about 30 months, and they are usually exposed for 24 to 27 months, beginning at weaning. The nominal life spans of nonrodents are much longer — for example, rabbit, 66 months; dog or pig, 120 months; and monkey, 184 months. Consequently, these animals are rarely subjected to life span exposures. However, a few literature examples have been noted of 7-year exposures for dogs.

When long-lived animals are chosen as second species to mice or rats in noncarcinogenic chronic toxicity studies, exposures of at least 10% of the projected life span of the animal are usually recommended. On this basis, dogs used as a second species in chronic toxicity tests should be exposed for at least a year. In the noncarcinogenic chronic toxicity publications analyzed in this study the typical exposure of dogs used as a second species was 2 years.

5.5 INTERIM SACRIFICE

Interim sacrifices are generally considered useful in the study of long-term toxic effects, pathogenesis, and reversible changes. Timely sampling may also provide valuable insights into specific clinical chemistry or organ function tests that may be required (Food Safety Council, 1978; National Academy of Sciences, 1977). For these reasons, some authorities recommend the inclusion of serial sacrifices in chronic toxicity tests (Fitzhugh, 1955, as cited in National Academy of Sciences, 1975; National Academy of Sciences, 1975; World Health Organization, 1978). However, other workers consider interim sacrifices a needless reduction in an otherwise limited number of test and control animals and recommend that interim sacrifices be avoided in chronic toxicity tests (Benitz, 1970). Obviously, extra animals could be taken initially to compensate for losses in size of the test and control groups through interim sacrifices. Frequently, however, similar information is available from previously performed interim sacrifices during subchronic tests or from earlier pharmacodynamic studies. The need for interim sacrifices in long-term studies thus varies from study to study and should be reassessed with each new study.

In actual practice, few investigators appear to make extensive use of interim sacrifices during chronic toxicity studies. Among more than 100 chronic toxicity publications examined during the preparation of this chapter, only 12 reported interim sacrifices, and only eight studies involved the examination of several animals during several intervals of the test period. Significant observations derived specifically from interim sacrifices were cited in only four of these reports.

In conclusion, the use of interim sacrifices in chronic toxicity testing is controversial. Some authorities recommend the practice as a valuable aid in the study of toxic effects, clinical chemistry, organ function tests, and pathogenesis. Other experts acknowledge the value of interim sacrifices for the purposes stated, but consider the practice a needless waste of the relatively limited number of test and control animals typically available in chronic toxicity tests. Much of the information provided by interim sacrifices in chronic toxicity tests can be provided by previously performed subchronic toxicity and pharmacodynamic studies. In actual practice, few workers appear to make extensive use of interim sacrifices during chronic toxicity studies. In the literature sample represented by the bibliography for this section interim sacrifices were reported in only 12 papers. Of these, only eight studies involved the examination of several animals during several intervals of the test period, and significant observations based on interim sacrifices were reported in only four papers. It thus appears that interim sacrifices are not a generally used, or needed, feature of chronic toxicity tests.

5.6 DATA COLLECTION AND EVALUATION

Long-term tests are unique research experiments that are only rarely amenable to standardized protocols (Food Safety Council, 1978). Nevertheless, the success of a well-planned chronic toxicity or carcinogenicity study strongly depends on adequate observations of the animal during the test period and on the efficacy of the ensuing pathological examinations. The following subsections address important aspects of clinical and pathological procedures essential to the successful execution of long-term tests.

5.6.1 Food Consumption and Body Weight

Growth and body weights are important indicators of adverse effects of the test material, but these data are not uniformly collected by some investigators. All animals should be weighed weekly during periods of rapid growth and at least monthly thereafter (Benitz, 1970; Food Safety Council 1978; Loomis, 1974; Magee, 1970; National Academy of Sciences, 1977; Sontag, Page, and Saffiotti, 1976; World Health Organization, 1978). Food consumption should also be monitored weekly to observe the effect of the test material on food intake and to provide an accurate determination of dose when the test material is administered in the diet. When the test compound is administered in drinking water, the weekly consumption rate of this material is also required for dose determinations.

5.6.2 Clinical and Laboratory Examinations

Careful clinical observation of test and control animals appears to be the most neglected area in experimental toxicology (Food Safety Council, 1978; World Health Organization, 1978). To remedy this situation and minimize the effects of extraneous factors on the course of chronic toxicity and carcinogenicity experiments, several authorities recommend that qualified employees observe the general physical condition, viability, and adverse behavioral changes of all test and control animals at least twice daily, 7 days a week (Arnold et al., 1977; Food Safety Council, 1978; Fox, 1977; National Academy of Sciences, 1977; World Health Organization, 1978). Animals with obvious life-threatening conditions should be isolated; those unlikely to survive an additional day should be sacrificed and necropsied to preserve tissues for histological examination. Each animal should be completely examined at least once a week by qualified personnel for unusual behavioral patterns, respiratory signs, bleeding, and palpable masses, as well as for abnormalities of the coat, eyes, mouth, teeth, nose, and ears (Arnold et al., 1977; National Academy of Sciences, 1977). In chronic toxicity studies, appropriate biochemical tests are also essential for detecting and assessing toxic effects at the clinical level, but no consensus exists regarding the extent or frequency of their use. Loomis (1974) recommended clinical blood chemistry tests, urinalysis, and blood cell counts for all animals at 6- to 12-week intervals, but stated "Routine special types of biochemical analyses of sample material, such as blood or urine, from apparently healthy animals probably are indicated only when there is reason to suspect that the chemical under investigation is capable of producing specific toxic effects for which biochemical methods are clinically of diagnostic value." A report of the National Academy of

Sciences (1977) suggests clinical chemistry tests "should be judiciously selected," based on preliminary or short-term toxicity tests, or on the known chemical characteristics of the test compound. The Academy report further states that a rigid sampling schedule is impractical and excessively costly. Other workers recommend "periodic clinical examinations" to identify and monitor the progression of specific abnormal findings (Food Safety Council, 1978). There appears to be general agreement, however, that clinical blood chemistry tests, blood cell counts, and urinalysis tests should be performed on all animals that become ill or show effects from exposure to the test material. Arnold et al. (1977), Fox (1977), and Street (1970) provide lengthy discussions of these tests, as well as extensive bibliographies of additional papers.

Less unanimity exists among investigators of chronic toxicity relative to prescheduled organ function tests. The minimum recommendation of the Food Safety Council (1978) includes tests for liver, kidney, and bone marrow function. The World Health Organization (1978) also recommends the use of organ function tests but concedes that "In general, these methods are not sufficiently standardized or reproducible to detect minor alterations in organ function." Benitz (1970) and the National Academy of Sciences (1977) questioned the benefits of prescheduled organ function tests because of their low sensitivity to organ damage. Instead of organ function tests, the latter workers recommended exposing extra animals that can be later sacrificed for complete gross and histopathological examinations. Other workers have also expressed reservations about the use of organ function tests (Peck, 1968). When organ function tests are included in chronic toxicity experiments, they are most often limited to liver, kidney, and thyroid studies in larger animals (National Academy of Sciences, 1977).

5.6.3 Pathological Examinations

Until recently, generally accepted procedures in long-term studies required the necropsy of all nonrodent test animals, but only a fraction of the total number of rodents (Benitz, 1970; Zbinden, 1973). Now, however, there appears to be general agreement among investigators that all animals that die during the study, or are subsequently killed, should be carefully necropsied (Arnold et al., 1977; Food Safety Council, 1978;

National Academy of Sciences, 1977; Sontag, Page, and Saffiotti, 1976; World Health Organization, 1978). Furthermore, current thinking stresses the importance of adequate gross examination of tissues prior to histologic sampling. In many instances, pertinent information missed during the macroscopic examinations of the test animal cannot be recovered in subsequent microscopic examinations of prepared tissues, even if the utmost care is used (Benitz, 1970). Consequently, the necropsy procedure should be considered one of the most important steps in the experimental protocol (Food Safety Council, 1978).

There is general agreement in the current literature that samples of all major organs and tissues should be taken from all test and control animals for macroscopic examination and fixation (Benitz, 1970; Food Safety Council, 1978; Loomis, 1974; National Academy of Sciences, 1977). No consensus exists, however, concerning which tissues should be examined microscopically. Early workers recommended microscopic examination of all obviously altered tissues plus an additional quantity that generally did not exceed 20 (Abrams, Zbinden, and Bagdon, 1965; Barnes and Denz, 1954). Other authorities left the microscopic observation of the supplementary tissues to the discretion of the pathologist in charge (Magee, 1970) or specified "complete and accurate" pathological examination without indicating more specific requirements (U.S. Food and Drug Administration, 1971). More recent guidelines require microscopic examination of all major tissues and gross lesions in all high dose and control animals, as well as grossly altered tissues in all other dose groups (Food Safety Council, 1978; National Academy of Sciences, 1977; Peck, 1974; World Health Organization, 1978). The National Cancer Institute guidelines for chronic toxicity tests are even more stringent: they require microscopic examination of approximately 40 tissues from all test and control animals, except positive controls which may be exempted from this requirement (Sontag, Page, and Saffiotti, 1976). Obviously, careful microscopic examination of all tissues is highly desirable from the experimental point of view; however, the incremental increase in information gained through such a practice must be balanced against the added economic, temporal, and labor costs incurred by such a requirement. According to recent estimates, the number of trained pathologists available to perform histologic examinations of tissues from tests required or expected under the Toxic Substances Control Act and other expanded government programs is insufficient if microscopic examinations of 40 or more tissues from all test and control animals are required (Page, 1977*b*).

In carcinogenicity testing, special emphasis is placed on the criteria used to classify the lesions found in microscopic examination (World Health Organization, 1978). The investigator should specify how the classification was done and what is implied by the terms *benign*, *malignant*, *neoplastic*, *preneoplastic*, and *hyperneoplastic*. Also, as discussed before, the pathologist should be aware of the type and location of the spontaneous tumors associated with the animal species/strain used (Magee, 1970). The value of proper pathology is especially great in carcinogenicity testing, since other evaluations are less frequently incorporated in the test design (Page, 1977*a*).

Microscopic analysis is usually performed on at least some tissues in most currently performed long-term tests. Histological examination of tissues was reported in 92% of the primary chronic toxicity studies referenced at the end of this chapter. Sixty percent of these papers indicated that "all major organs" or "all major organs and tissues" were so examined. Ten percent of the references mentioned histological examination of six or fewer specific organs, and 14% referred to microscopic examination of only one or two specific organs. In 8% of the references, organs examined microscopically were not identified. In a similar number of papers, histological examination of tissues was apparently not performed at all.

5.6.4 Conclusions

The successful execution of chronic toxicity and carcinogenicity tests requires careful collection and evaluation of clinical and pathological data. All animals should be observed twice daily, 7 days a week, by qualified personnel for changes in physical condition, viability, and behavior. Body weight should be measured weekly during periods of rapid growth and at least monthly thereafter. Food consumption should be similarly measured, except when the test material is administered in the diet; then, weekly measurements are required for dose determinations.

Each animal should be examined at least weekly for unusual behavioral patterns, respiratory signs, excretory products, and palpable masses, as well as other abnormalities. In noncarcinogenic chronic tests there is general agreement that animals showing obvious signs of illness should receive blood chemistry and urinalysis tests, but no consensus exists concerning routine tests for healthy animals. Even less unanimity exists relative to prescheduled organ function tests, which many authorities regard as insufficiently standardized and reproducible to detect minor alterations in organ function. When performed, these tests are generally limited to the kidney and liver function of larger, nonrodent test animals.

There is general agreement among experts that samples of all major organs and tissues should be taken from all test and control animals for macroscopic examination and fixation, but no consensus exists concerning which tissues should be examined microscopically. Obviously, careful microscopic examination of all sample tissues is highly desirable from the experimental point of view, but, according to recent estimates, there are too few trained pathologists to perform histologic examinations of tissues from tests required or expected under the Toxic Substances Control Act and other expanded government programs if microscopic examinations of 40 or more tissues from all test and control animals are required. It thus appears that, at least for the next few years, limitations must be placed on (1) the number of long-term tests performed, if total histologic tissue analysis of all animal samples is required, (2) the number of animals to be examined histologically in each long-term test, or (3) the number of tissues per animal to be examined histologically. In the literature analyzed in this study, most workers performed histological examinations of "all major organs and tissues" of high dose and control animals, plus any other tissues exhibiting gross lesions. In most thorough studies, "all major organs and tissues" correspond to approximately 30 histological samples. The analysis of this number of samples from the high dose and control groups is recommended as the minimum acceptable level for histological sampling at the present time.

5.7 SHORT-TERM TESTS FOR CARCINOGENICITY

Because of time and cost requirements, application of the lifetime rodent bioassay to the screening of all potential carcinogens is practically impossible. Short-term in vitro screening procedures have been developed that provide limited information concerning the potential carcinogenicity of chemicals, under reasonable time and cost constraints. Such tests, designed to detect genotoxic activity or morphological transformation, include the following: the Ames Salmonella/microsome assay; the polA+/polA- assay (differential toxicity in E. coli); "Rec" differential toxicity in Bacillus subtilis; mouse lymphoma assay (mutation in cultured mouse cells); and assays for unscheduled DNA synthesis, in vitro cell transformation, sex-linked recessive lethal mutation in Drosophila, sister chromatid exchange, and in vitro chromosome aberrations. These assays are being examined closely in the GENE-TOX workshop series (sponsored by the U.S. Environmental Protection Agency) and therefore will not be included in this discussion of short-term tests for carcinogenicity. For evaluation of advantages and limitations of the assays mentioned above, see Brusick (1978), Bridges (1976), and Stoltz et al. (1974).

The discussion of short-term tests will be limited to brief descriptions of in vivo (or in vivo-in vitro) protocols which allow for metabolic activation of chemical carcinogens in the host animal. These tests are designed to shorten tumor induction time or to detect cellular alterations or markers that appear shortly after exposure to carcinogens.

5.7.1 Embryo Homograft

A promising technique for producing tumors with chemicals in a relatively short time was introduced by Peacock in 1962 and was discussed in more detail by Peacock and Dick (1963). Using BALB/c mice, tissues from various organs of embryos were implanted surgically into the thigh muscle of adults of the same strain. Skin, lung, stomach, and urinary bladder yielded 100% growing implants. Kidney, adrenal, thymus, and spleen implants were sometimes successful, but liver and brain tissues failed to grow. When small quantities of each of 13 solid polycyclic hydrocarbons were introduced along with the tissue, malignant tumors formed in the implants. The quantity of carcinogen required to induce tumors was estimated to be no more than 150 μg, and the tumors induced were mainly squamous cell carcinomas (with some sarcomas and lung adenomas); however, the incidence and types of embryo tissue affected varied with each hydrocarbon. Only those hydrocarbons previously reported to be carcinogens (by any method) produced tumors during the time of observation. The results were obtained in 16 weeks. The surgical technique used for implantation introduced the problem of possible effect of the carcinogen on the healing process, but Davies, Major, and Aberdeen (1971), using a nonsurgical implantation technique, were able to induce adenomas in embryonic lung tissues exposed to 26 μg of 3,4-benzo(α)pyrene (BAP) or 1,2,5,6-dibenzanthracene. Twenty percent of the implants exposed to benzo(α)pyrene and 62% of the implants exposed to dimethylbenzanthracene developed adenomas in 16 weeks. The technique lacks quantitation, but that disadvantage can perhaps be eliminated by in vitro exposure of organ cultures to accurately measured amounts of carcinogen. For example, lung explants exposed to 20-methylcholanthrene, 3,4-benzo(α)pyrene, or 1,2,5,6-dibenzanthracene and implanted subcutaneously into a host animal developed adenomas (some as early as 3 months later), as demonstrated by Laws and Flaks (1966) and Davies, Major, and Aberdeen (1970).

5.7.2 Site Transfer

Homburger and Baker (1967) described studies in which mice and hamsters received subcutaneous injections of 25 γ and 500 γ, respectively, of 3,4,9,10-dibenzopyrene. At various times after injection, the injection sites were removed. Four sites were pooled, minced, and injected subcutaneously into recipients of the same age, sex, and strain. The test animals were checked weekly for tumor formation. Control mice were injected with a carcinogen, injection sites being left intact, and were also checked for tumor development. One group of hamsters was also injected with carcinogen and the injection sites were removed at various times for histological study.

Homburger and Baker found that 10% of the secondary host mice developed palpable tumors by 10 weeks, whereas 14 to 15 weeks were required for 10% of the primary hosts to develop palpable tumors. In the hamster study, tumors palpable 4 weeks after transfer of the injection sites later turned out to be malignant fibrosarcomas. These particular injection sites had been transferred 2 days after carcinogen injection.

RNA-loaded atypical fibroblasts appeared at the site of administration soon after injection and increased in number until a fibrosarcoma was formed. Homburger suggested that the fibroblasts represent premalignant cells which, if left in the original host, slowly develop into a fibrosarcoma but which, when transferred to a secondary host, express their malignant behavior more readily. Homburger and Baker proposed that (by using susceptible inbred strains of hamsters, by developing harvesting methods for selective collection of fibroblasts, and by transfer of such cells into fresh hamsters) a test is theoretically possible requiring less than 1 month for the demonstration of carcinogenic potency in vivo in adult mammalian cells that yield tumors as end points.

5.7.3 Partial Hepatectomy

Using the 1938 study of Alexander Haddow as a starting point, Solt, Medline, and Farber (1977) developed a new model for the sequential analysis of liver carcinogenesis. In their description of this model Solt et al. stated that cancer cells will emerge under conditions that would inhibit or impair the growth of normal cells. Hyperplastic liver lesions induced by the administration of hepatocarcinogens are resistant to other hepatocarcinogens and hepatotoxins that impair the growth of surrounding normal liver cells.

Single doses of diethylnitrosamine eventually produce liver cancer, and during the first week after exposure diethylnitrosamine-induced nodules arise on the surface of the liver. The model proposed by Solt, Medline, and Farber (1977) contains the following features: initiation by diethylnitrosamine, selective growth inhibition of normal cells by 2-acetylaminofluorene and growth stimulation of carcinogen-altered hepatocytes by partial hepatectomy. The test can be completed in 4 weeks. The procedure in male Fischer 344 rats was as follows: (1) the rats were injected intraperitoneally with 200 mg/kg diethylnitrosamine, (2) after a 2-week recovery period the animals were placed on a basal diet containing 0.02% acetylaminofluorene, and (3) after 1 week on the 2-acetylaminofluorene diet the rats were subjected to two-thirds partial hepatectomy. The animals were maintained on the carcinogen diet after surgery until 24 h before sacrifice or for one more week. The liver nodules continued to proliferate vigorously under these experimental conditions, were characterized histologically by the investigators, and were identified as precursor lesions for some hepatocellular carcinomas.

In experiments designed to study the cellular components involved in hepatocarcinogenesis and the progression of such cells to malignancy, Laishes and Farber (1978) isolated the carcinogen-altered hepatocyte populations from the livers of treated rats. The cells, some bearing a γ-glutamyl transpeptidase (γ-GT) marker, were transferred to the livers of syngeneic host rats. Selective proliferation of the γ-GT-positive hepatocytes was then stimulated in the host rat liver by partial hepatectomy, while proliferation of γ-GT-negative host hepatocytes was inhibited by dietary administration of 2-acetylaminofluorene. The system is quantitative and rapid — macroscopic foci were observed within 10 days of cell transfer. In a recent report Laishes, Fink, and Carr (1980) described in vitro purification of the carcinogen-altered cell populations isolated from rats treated with the diethylnitrosamine/2-acetylaminofluorene/partial hepatectomy regime. Primary hepatocyte monolayer cultures from carcinogen-treated and untreated rats were exposed for 24 h to acetylaminofluorene, to its derivatives, or to certain chemicals known to be cytocidal but nonhepatocarcinogenic. Acetylaminofluorene (AAF), its derivatives N-OH-AAF and N-acetoxy-AAF, and three chemotherapeutic drugs — methotrexate, cyclohexamide, and adriamycin — were found to be selectively cytocidal to normal rat hepatocytes. However, the hepatocyte phenotypes which developed during hepato-carcinogenesis were highly resistant to the effects of the chemicals. Studies are under way in which cells selected in vitro will be transferred to syngeneic hosts for determination of colony-forming ability.

Tatematsu et al. (1977) adapted the system of Solt, Medline, and Farber (1977) as an in vivo screening test for hepatocarcinogens. Their procedure was as follows: (1) rats were injected intraperitoneally with

200 mg diethylnitrosamine per kg of body weight; (2) two weeks later the animals were started for 2 weeks on the test chemical in the diet or drinking water; (3) after 1 week of administration of the test chemical partial hepatectomy was performed; and (4) one week later the rats were killed. Various control groups were established. The end points of the experiment were the number of liver hyperplastic nodules and the histochemical observation of γGT, acid phosphatase (acid-Pase), glucose-6-phosphatase (G-6-Pase), and adenosine triphosphatase (ATPase) activity. The chemicals tested included *N*-2-fluorenylacetamide, 3'-methyl-4-(dimethylamino)azobenzene, dimethylnitrosamine, diethylnitrosamine, DL-ethionine, quinoline, 5,7-dibromo-8-hydroxy-quinoline, 8-hydroxyquinoline, or 8-nitroquinoline. The first six chemicals, known carcinogens, in combination with diethylnitrosamine and partial hepatectomy, induced statistically significantly more hyperplastic liver nodules than did diethylnitrosamine and partial hepatectomy alone. γ-GT activity, not demonstrated in normal hepatocytes or after partial hepatectomy, was seen in hyperplastic nodules and in the epithelium of bile ducts and ductules in the portal area. Acid-Pase and G-6-Pase were usually decreased, and ATPase was usually absent in hyperplastic areas. Thus, the presence of γ-GT and absence of ATPase are good markers for detection of hyperplastic nodules.

Tatematsu et al. suggested further evaluation of the system, particularly to eliminate the possibility of a noncarcinogen acting as a selective growth inhibitor. They suggested that it may be useful as an in vivo short-term test in conjunction with in vitro assay systems and together these tests would provide a secondary screening for chemicals to be studied in long-term in vivo studies. Laishes, Fink, and Carr (1980) have recently demonstrated that three noncarcinogenic chemotherapeutic agents are selectively cytocidal to normal cells in vitro. (See above.)

5.7.4 Alkaline Elution

Petzold and Swenberg (1978) described a procedure for detecting single strand breaks in DNA of tissues taken from a wide range of organs of rats following in vivo exposure to known carcinogens of several classes.

Newborn rats were injected intraperitoneally with (^{3}H)dThd (tritiated deoxythymidine) for 3 weeks. The animals were exposed to chemicals for various periods of time, and tissues were then removed and homogenized. The (^{3}H)DNA elution was carried out as described by Swenberg, Petzold, and Harbach (1976). Increased alkaline elution was expressed as the increase in elution of treated rats over that of vehicle control rats.

Twenty-three chemicals were tested and the following organs were evaluated for DNA damage: liver, lung, kidney, brain, thymus, duodenum stomach, bone marrow, and mammary gland. The tissues tested were from target and nontarget organs, and there was a high degree of correlation between tissues with increased elution after in vivo exposure to carcinogens and target organs for tumor susceptibility. For example, the hepatocarcinogens dimethylnitrosamine, diethylnitrosamine, 2-acetylaminofluorene, and *N*-hydroxyacetylaminofluorene caused the greatest elution in liver preparations. Less correlation was seen with chemicals not requiring metabolic activation. The alkaline elution assay is rapid (not as time-consuming as the alkaline sucrose gradient technique) and is reproducible.

Petzold and Swenberg (1978) recommended the alkaline elution test as a confirmation of in vitro tests when the carcinogenic potential of a compound is unknown. It can help identify target organs for procarcinogens and can be utilized to assess DNA damage in organs that are subject to toxic changes.

5.7.5 α-Fetoprotein in Serum

α-Fetoprotein has been found in animals bearing chemically induced liver tumors. Watabe (1971) described the appearance of α-fetoprotein in the serum of rats after the start of feeding of 4-dimethylaminoazobenzene. The protein was detected in the sera of approximately 20% of 41 rats by as early as 3 weeks and in 76% of the rats by 6 weeks. The serum protein disappeared at 11 to 12 weeks and reappeared at 13 weeks in 27 of 33 rats; 26 of these 27 rats developed hepatomas. Ninety-one percent of the rats that developed "early" α-fetoprotein levels had tumors after 19 weeks.

Serum levels of the protein increased from 2 to 4 mg/dL in the early stage up to 60 to 100 mg/dL in the later stage. Watabe suggested that the early protein peak could be attributed to chemical toxicity not related to carcinogenicity.

In a later study Kroes, Williams, and Weisburger (1973) tested the effects of different dose levels of several hepatotoxins and hepatocarcinogens on the early appearance of α-fetoprotein. They found that high levels of liver carcinogen were required to induce the "early" peak at 2 to 5 weeks. The protein was not detected in the sera of rats treated with hepatotoxins. Kroes suggested possibly using the α-fetoprotein assay as a short-term test to distinguish between hepatotoxins and hepatocarcinogens.

5.7.6 Strain Susceptibility

Differences in the responses of various strains of animals to chemical carcinogenesis are discussed in Sect. 5.2.2. Selection of sensitive strains of test animals can shorten tumor induction time considerably. The following are examples of such instances.

5.7.6.1 Hamster Fibrosarcoma System — Homburger and Hsuch (1970) tested the susceptibility of inbred strains of hamsters to tumor induction with 7,12-dimethylbenz(*a*)anthracene. A dose of 500 μg of the chemical, injected subcutaneously, gave rise to subcutaneous fibrosarcomas in a relatively short time. The 15.16 line was the most sensitive, with males and females developing tumors having average induction times of 10 and 9 weeks, respectively. The 82.73 line was the least sensitive, with males and females developing tumors having average induction times of 18 and 15 weeks, respectively.

5.7.6.2 Rat Mammary Tumor System — Huggins, Grand, and Brillantes (1961) reported preferential mammary tumor induction in female Sprague-Dawley rats following single oral doses of polynuclear hydrocarbons.

All rats injected with 20 mg or 15 mg of 7,12-dimethylbenzanthracene developed mammary tumors; the tumor induction time and the number of active centers were dose related. Doses of 2 to 100 mg of 3-methylcholanthrene induced tumors in 10% to 100% of the animals, and the effect was dose related; induction times and the numbers of active centers were not. Doses of 100 mg and 500 mg of 2-acetylaminofluorene produced tumors in 30% of the animals, and only the induction times were dose related.

Hormonal treatments suppressed the development of mammary tumors caused by 3-methylcholanthrene. 7,12-Dimethylbenzanthracene was the most effective compound for the induction of mammary tumors (100% tumors in 43 days).

In a later study, Huggins, Grand, and Fukunishi (1964) demonstrated in the mammary tumor system that intravenous injection of a compound in three equal doses was more effective in inducing tumors than the sum of the doses given as a single injection. The latent period was shorter, and more active centers per animal were produced, although in both cases 100% of the rats developed tumors.

Griswold et al. (1968) tested 35 compounds using the mammary tumor induction system. Ten doses of the maximum tolerated dose were administered to each animal, and the experiment was terminated 9 months later. (The duration was increased to 9 months for added sensitivity.) Multiple doses increased the number of tumor-bearing animals and the number of tumors per rat; however, multiple doses were no more effective than the single dose for determining general carcinogenicity of a compound. The mammary tumor assay is a sensitive test for detection of polynuclear aromatic hydrocarbons, polycyclic nitro and amino derivatives, and certain heterocyclic compounds. The investigators recommend the test for rapid screening of certain potential carcinogens.

5.7.6.3 Mouse Lung Adenoma System — The lung adenoma system was first utilized as a bioassay of chemicals for carcinogenic potential by Andervont and Shimkin (1940). In an exhaustive review of the lung adenoma bioassay system, Shimkin and Stoner (1975) outlined the now standardized procedure:

1. A susceptible inbred strain of mouse is selected, usually strain A. Weanlings are generally used, and both sexes should be included in initial bioassays.

2. The maximum tolerated dose of the chemical must be determined, and that dose is administered three times per week for 8 weeks. The second dose level is one-half the first and that of the third group is one-fifth the first. Chemicals are generally injected intraperitoneally, but other routes can be used more effectively with certain chemicals.
3. Animals can be sacrificed in 12 to 24 weeks. The nodules appearing on the surface of the lung are counted and are usually statistically evaluated; however, most tests are considered positive if the number of lung tumors per mouse is increased by one or more, if there is a dose-response relationship, and if the mean number of lung tumors in the vehicle and untreated controls is that usually found.

The advantages of the system are that it is a rapid, convenient, and quantitative procedure. The criticisms are that the adenoma does not have a counterpart in the human and that the tumors represent an increase in incidences of spontaneous tumors instead of an inductive process.

5.7.7 The Sebaceous Gland Test

The sebaceous gland suppression test has been suggested as a possible screening test for skin carcinogens (Brune, 1977; National Academy of Sciences, 1975). Exposure of the skin to polycyclic hydrocarbons leads to thickened epidermis and atrophy of the sebaceous glands in a matter of days. However, false positive results have been obtained with the test (Weisburger, 1976), making it subject to criticism.

5.7.8 Host-Mediated In Vivo-In Vitro Assay

DiPaolo et al. (1973) described a combination in vivo-in vitro bioassay for carcinogenicity utilizing transplacental exposure of Syrian golden hamster embryos. Pregnant females were injected intraperitoneally with test chemicals at 10 to 11 days gestation. The embryos were excised at day 13 of gestation, and cells from the embryos were cultured in Dulbeccos' modified Eagle's or F-12 medium supplemented with 10% fetal bovine serum. Cells were passed every 4 to 6 days from secondary cultures. Colonies, formed by cells transferred to feeder layers, were scored for morphologic transformation and were further checked for tumorigenicity by animal innoculation. The cells were harvested and were injected subcutaneously into hamsters, which were watched closely for subsequent tumor development. Sarcomas were produced in 3 to 16 weeks. Twelve known chemical carcinogens, of which several were known to be inactive in in vitro tests, were tested in this system, and all produced neoplastic transformation; five noncarcinogens did not. The results were reproducible, the assay seems to eliminate false negative results that occur because of the requirement for metabolic activation, and spontaneous transformation does not occur.

5.7.9 Conclusions

Short-term tests for carcinogenicity are not intended to replace the conventional lifetime studies. However, these types of assays could be valuable tests in the screening of chemicals for carcinogenicity or, more precisely, for lack of carcinogenic potential. A battery of short-term tests could be designed to identify safe compounds so that they can be eliminated from further testing.

The procedures described above can be completed in from 3 weeks (appearance of α-fetoprotein in the serum) to 3 to 4 months (the time required for the detection of actual tumors, as in the lung adenoma assay and the embryo homograft technique). Because exposure of cells to chemicals occurs in vivo, these particular assays have the additional advantage of allowing for metabolic activation. However, in order to determine the best application of in vivo short-term assays to the screening of chemicals for carcinogenicity, further evaluation of such tests is necessary, and their limits of sensitivity must be established.

REFERENCES FOR SECTION 5

Abrahms, W., G. Zbinden, and R. Bagdon. 1965. Investigative Methods in Clinical Toxicology. J. New Drugs 5:199-207.

Adamson, R. H., R. W. Cooper, and R. W. O'Gara. 1970. Carcinogen-Induced Tumors in Primitive Primates. J. Natl. Cancer Inst. 45:555-560.

Adamson, R. H., P. Correa, and D. W. Dalgard. 1973. Occurrence of a Primary Liver Carcinoma in a Rhesus Monkey Fed Aflatoxin B_1. J. Natl. Cancer Inst. 50(2):549-553.

Agthe, C., H. Garcia, P. Shubik, L. Tomatis, and E. Wenyon. 1970. Study of the Potential Carcinogenicity of DDT in the Syrian Golden Hamster. Proc. Soc. Exp. Biol. Med. 134:113-116.

Akamatsu, Y., and B. Barton. 1974. Neoplasms and Amyloidosis in Strains of Mice Treated with 3-Methylcholanthrene. J. Natl. Cancer Inst. 52:377-385.

Alfred, L. J., A. Globerson, Y. Berwald, and R. T. Prehn. 1964. Differential Toxicity Response of Normal and Neoplastic Cells *In Vitro* to 3,4-Benzopyrene and 3-Methylcholanthrene. Br. J. Cancer 18:159-164.

Allen, M. J., E. Boyland, C. E. Dukes, E. S. Horning, and J. G. Watson. 1957. Cancer of the Urinary Bladder Induced in Mice with Metabolites of Aromatic Amines and Tryptophan. Br. J. Cancer 11:212-230.

Andervont, H. B. 1950. Induction of Hemangio-endotheliomas and Sarcomas in Mice with *O*-Aminoazotoluene. J. Natl. Cancer Inst. 10:927-941.

Andervont, H. B. 1958. Induction of Hepatomas in Strain C3H Mice with 4-*O*-Tolylazo-*O*-Toluidine and Carbon Tetrachloride. J. Natl. Cancer Inst. 20:431-438.

Andervont, H. B., and T. B. Dunn. 1962. Occurrence of Tumors in Wild House Mice. J. Natl. Cancer Inst. 28:1153-1163.

Andervont, H. B., H. G. Grady, and J. E. Edwards. 1942. Induction of Hepatic Lesions, Hepatomas, Pulmonary Tumors and Hemangio-endotheliomas in Mice with *O*-Aminoazotoluene. J. Natl. Cancer Inst. 3:131-153.

Andervont, H. B., and M. B. Shimkin. 1940. Biologic Testing of Carcinogens. II. Pulmonary-Tumor-Induction Technique. J. Natl. Cancer Inst. 1:225-239.

Andrianova, M. 1971. Transplacental Action of 3-Methylcholanthrene and Benz(*a*)pyrene on Four Generations of Mice. Bull. Exp. Biol. Med. (USSR) 71(1):677-680.

Argus, M. F., and C. Hoch-Ligeti. 1963. Induction of Malignant Tumors in the Guinea Pig by Oral Administration of Diethylnitrosamine. J. Natl. Cancer Inst. 30:533-551.

Arnold, D., S. Charbonneau, Z. Zawidzka, and H. Grice. 1977. Monitoring Animal Health During Chronic Toxicity Studies. J. Environ. Pathol. Toxicol. 1:227-239.

Axelrad, A., and H. van der Gaag. 1962. Susceptibility to Lymphoma Induction by Gross' Passage A Virus in C3Hf/Bi Mice of Different Ages: Relation to Thymic Cell Multiplication and Differentiation. J. Natl. Cancer Inst. 28:1065-1094.

Baba, T., and S. Takayama. 1961. Influence of *p*-Dimethylamine-Azobenzene (DAB) Pretreatment in Neonatal Stage on the DAB Heptacarcinogenesis in Adult Rats, with Special Reference to Sex Difference in the Carcinogenesis. Gann 52:73-82.

Barnes, J., and F. Denz. 1954. Experimental Methods in Determining Chronic Toxicity. Pharmacol. Rev. 6:191-242.

Beckers, A., and H. Bazin. 1978. Incidence of Spontaneous Ileocecal Immunocytomas in Hybrids of LOU/C Rats and Rat Strains with Low Spontaneous Tumor Incidence (brief communication). J. Natl. Cancer Inst. 60(6):1505-1508.

Benitz, K. F. 1970. Measurement of Chronic Toxicity. In: Methods in Toxicology, G. E. Paget, ed. F. A. Davis Company, Philadelphia. pp. 82-131.

Berenblum, I., L. Boiato, and N. Trainin. 1966. On the Mechanism of Urethane Leukemogenesis in Newborn C57BL Mice. II. Influence of Thymectomy and of Subsequent Thymus Reimplantation. Cancer Res. 26:361-363.

Bhisey, R. A., and S. M. Sirsat. 1975. Histological Changes in the Mouse Skin After One Subcutaneous Injection of 20-Methylcholanthrene. Indian J. Cancer 12:430-438.

Bielschowsky, F., and W. S. Bullough. 1949. Epidermal Mitotic Activity and the Induction of Skin Tumors in Mice. Br. J. Cancer 3:282-285.

Bonser, G. M., E. Boyland, E. R. Busby, D. B. Clayson, P. L. Grover, and J. W. Jull. 1963. A Further Study of Bladder Implantation in the Mouse as the Means of Detecting Carcinogenic Activity: Use of Crushed Paraffin Wax or Stearic Acid as the Vehicle. Br. J. Cancer 17:127-136.

Bonser, G. M., D. B. Clayson, J. W. Jull, and L. N. Pyrah. 1952. The Carcinogenic Properties of 2-Amino-1-Naphthol Hydrochloride and Its Parent Amine 2-Naphthylamine. Br. J. Cancer 6:412-424.

Boorman, G. A., and C. F. Hollander. 1974. High Incidence of Spontaneous Urinary Bladder and Ureter Tumors in the Brown Norway Rat (brief communication). J. Natl. Cancer Inst. 52(3):1005-1008.

Boyd, E. 1968. Predictive Drug Toxicity: Assessment of Drug Safety Before Human Use. Can. Med. Assoc. J. 98:278-293.

Braun, W., L. Sung, D. Keyes, and R. Kociba. 1977. Pharmacokinetic and Toxicological Evaluation of Dogs Fed 1,2,4,5-Tetrachlorobenzene in the Diet for Two Years. Toxicol. Appl. Pharmacol. 41:218-219.

Bridges, B. A. 1976. Short Term Screening Tests for Carcinogens. Nature 261:195-200.

Brune, H.F.K. 1977. Experimental Results with Percutaneous Applications of Automobile Exhaust Condensates in Mice. In: Air Pollution and Cancer in Man, U. Mohr, D. Schmahl, and L. Tomatis, eds. International Agency for Research on Cancer, Lyon, France. pp. 41-47.

Brusick, D. J. 1978. The Role of Short-Term Testing in Carcinogen Detection. Chemosphere No. 5:403-417.

Burek, J. D., and C. F. Hollander. 1977. Incidence Patterns of Spontaneous Tumors in BN/Bi Rats. J. Natl. Cancer Inst. 58(1):99-105.

Butler, W. H. 1979. [illegible] Toxicol. Environ. Health 5:161-162.

Cameron, G. R., and K. K. Cheng. 1951. Failure of Oral DDT to Induce Toxic Changes in Rats. Br. Med. J. 2:819-821.

Campbell, J. S., Y. H. Yang, and J. D. Bolton. 1965. Murine Lung Adenomas Induced by Intravaginal Carcinogen. Arch. Path. 79:588-594.

Cardesa, A., P. Pour, J. Althoff, and U. Mohr. 1974. Effects of Intraperitoneal Injections of Dimethyl- and Diethylnitrosamine, Alone or Simultaneously on Swiss Mice. Z. Krebscorsch. 82:233-238.

Chieco-Bianchi, L., G. De Benedictis, G. Tridente, and L. Fiore-Donati. 1963. Influence of Age on Susceptibility to Liver Carcinogenesis and Skin Initiating Action by Urethane in Swiss Mice. Br. J. Cancer 17: 672-680.

Clapp, N. K., R. L. Tyndall, and J. A. Otten. 1971. Differences in Tumor Types and Organ Susceptibility in BALB/c and RF Mice Following Dimethylnitrosamine and Diethylnitrosamine. Cancer Res. 31:196-198.

Clark, D. 1977. Long-Term Inhalation Toxicology Studies. In: Current Approaches in Toxicology, B. Ballantyne, ed. John Wright and Sons, Bristol. pp. 105-114.

Crocker, T. T., J. E. Chase, S. A. Wells, and L. L. Nunes. 1970. Preliminary Report on Experimental Squamous Carcinoma of the Lung in Hamsters and in a Primate. In: Morphology of Experimental Respiratory Carcinogenesis, P. Nettesheim, M. G. Hanna, Jr., and J. W. Deatheridge, Jr., eds. Oak Ridge, Tenn., USAEC Division of Technical Information. pp. 317-328.

Dale, M. M., G. C. Easty, R. Tchao, H. Desai, and M. Andjargholi. 1973. The Induction of Tumors in the Guinea Pig with Methylcholanthrene and Diethylnitrosamine and Their Propogation *In Vivo* and *In Vitro*. Br. J. Cancer 27:445-450.

Davies, R. F., I. R. Major, and E. R. Aberdeen. 1970. Pulmonary Adenomata Induced by Carcinogen Treatment in Organ Culture. Influence of Increasing Amounts of Carcinogen. Br. J. Cancer 24:785-787.

Davies, R. F., I. R. Major, and E. R Aberdeen. 1971. The Induction of Adenomata in Mouse Lung Homografts by Chemical Carcinogens. Br. J. Cancer 25:565-567.

Davis, B. R., J. K. Whitehead, M. E. Gill, P. N. Lee, A. D. Butterworth, and F.J.R. Roe. 1975. Response of Rat Lung to 3,4-Benzpyrene Administered by Intratracheal Instillation in Infusine With or Without Carbon Black. Br. J. Cancer 31:443-452.

De Benedictis, G., G. Maiorano, L. Chieco-Bianchi, and L. Fiore-Donati. 1962. Lung Carcinogenesis by Urethane in Newborn, Suckling, and Adult Swiss Mice. Br. J. Cancer 16:686-689.

Deichmann, W. B., M. Keplinger, F. Sala, and E. Glass. 1967. Synergism Among Oral Carcinogens. IV. The Simultaneous Feeding of Four Tumorigens to Rats. Toxicol. Appl. Pharmacol. 11:88-103.

Deichmann, W. B., and J. L. Radomski. 1963. The Carcinogenicity and Metabolism of 2-Naphthylamine and Related Compounds. Ind. Med. Surg. 32:161.

Deichmann, W. B., J. L. Radomski, E. Glass, W.A.D. Anderson, M. M. Coplan, and F. M. Woods. 1965. Synergism Among Oral Carcinogens. III. Simultaneous Feeding of Four Bladder Carcinogens to Dogs. Ind. Med. Surg. 34:640-649.

Della Porta, G. 1968. Use of Newborn and Infant Animals in Carcinogenicity Testing. Food Cosmet. Toxicol. 6:243-252.

Della Porta, G., L. Kolb, and P. Shubik. 1958. Induction of Tracheobronchial Carcinomas in the Syrian Golden Hamster. Cancer Res. 18:592-597.

Della Porta, G., M. Rappaport, U. Saffiotti, and P. Shubik. 1956. Induction of Melanotic Lesions During Skin Carcinogenesis in Hamsters. Arch. Pathol. 61:303-313.

Della Porta, G., and B. Terracini. 1969. Chemical Carcinogenesis in Infant Animals. Prog. Exp. Tumor Res. 11:334-363.

DiPaolo, J. A., R. L. Nelson, P. J. Donovan, and C. H. Evans. 1973. Host Mediated *In Vivo - In Vitro* Assay for Chemical Carcinogenesis. Arch. Pathol. 95:380-385.

Diwan, B. A., and H. Meier. 1974. Strain- and Age-Dependent Transplacental Carcinogenesis by 1-Ethyl-1-Nitrosourea in Inbred Strains of Mice. Cancer Res. 34:764-770.

Doell, R., and W. Carnes. 1962. Urethane Induction of Thymic Lymphoma in C57BL Mice. Nature 194:588-589.

Durham, W. F., P. Ortega, and W. J. Hays, Jr. 1963. The Effect of Various Dietary Levels of DDT on Liver Function, Cell Morphology and DDT Storage in the Rhesus Monkey. Arch. Int. Pharmacodyn. 141:111-129.

Dyer, H., M. Kelly, and R. O'Gara. 1966. Lack of Carcinogenic Activity and Metabolic Fate of Fluorenylacetamide in Monkeys. J. Natl. Cancer Inst. 36:305-322.

Epstein, S., J. Andrea, and H. Jaffe. 1967. Carcinogenicity of the Herbicide Maleic Hydrazide. Nature (London) 215:1388-1390.

Fancher, O. 1978. Species Selection and Animal Models for Toxicologic Studies. Clin. Toxicol. 12(2):239-247.

Farber, E. 1963. Ethionine Carcinogenesis. Adv. Cancer Res. 7:383-474.

Fare, G. 1966. Rat Skin Carcinogenesis by Topical Applications of Some Azo Dyes. Cancer Res. 26:2406-2408.

Federal Register. 1978. Proposed Guidelines for Registering Pesticides in the U.S. Hazard Evaluation: Humans and Domestic Animals. 43FR(163): 37336-37403.

Federal Register. 1979. Proposed Health Effects Tests Standards for Toxic Substances Control Act Test Rules. 44FR(91):27334-27375.

Flaks, A. 1965. The Effect of 9,10-Dimethyl-1,2-Benzanthracene on Young Mice of Low and High Cancer Strain. Br. J. Cancer 19:547-550.

Flaks, A. 1966. Test for Carcinogenesis of Cigarette Tobacco Smoke Condensate Using Young Strong A and C57BL Mice. Br. J. Cancer 20:145-147.

Food Safety Council. 1978. Proposed System for Food Safety Assessment. Food Cosmet. Toxicol. 16, Suppl. 2:1-136.

Fox, J. G. 1977. Clinical Assessment of Laboratory Rodents on Long-Term Bioassay Studies. J. Environ. Path. and Toxicol. 1:199-226.

Friedman, L. 1973. Problems of Evaluating the Health Significance of the Chemicals Present in Foods. In: Pharmacology and the Future of Man, Proc. 5th Int. Congr. Pharmacology, San Francisco, 1972, Vol. 2. Karger, New York. pp. 30-41.

Fugmann, R. A., J. C. Anderson, R. L. Stolfi, and D. S. Martin. 1977. Comparison of Adjuvant Chemotherapeutic Activity Against Primary and Metastatic Spontaneous Murine Tumors. Cancer Res. 37:496-500.

Gak, J. C., C. Graillot, and R. Truhaut. 1976. Use of the Golden Hamster in Toxicology (Part II of two parts). Lab. Anim. Sci. 26(2):274-280.

Gargus, J., O. Paynter, and W. Reese, Jr. 1969. Utilization of Newborn Mice in the Bioassay of Chemical Carcinogens. Toxicol. Appl. Pharmacol. 15:552-559.

Gilbert, C., J. Gillman, P. Loustalot, and W. Lutz. 1958. The Modifying Influence of Diet and the Physical Environment on Spontaneous Tumor Frequency in Rats. Br. J. Cancer 12:565-593.

Glucksmann, A., and C. Cherry. 1971. Sex Difference and Carcinogenic Dosage in the Induction of Neoplasms in the Salivary Glands of Rats. Br. J. Cancer 25:212-224.

Goerttler, K., and H. Lohrke. 1977. Diaplacental Carcinogenesis: Tumor Localization and Tumor Incidence in NMRI Mice After Diaplacental Initiation [illegible] Urethane and Postnatal Promotion With the Phorbol Ester TPA in a Modified 2-Stage Berenblum/Mottram Experiment. Virchos Arch. A. Path. Anat. and Histol. 376:117-132.

Golberg, L., ed. 1974. Carcinogenesis Testing of Chemicals. The Chemical Rubber Company, Cleveland, Ohio. 144 pp.

Golberg, L. 1975. Safety Evaluation Concepts. J. Assoc. Off. Anal. Chem. 58(4):635-644.

Goldenthal, E. 1968. Current Views on Safety Evaluation of Drugs. U.S. Food and Drug Administration Papers May:13-18.

Goldwater, L. J., A. J. Rosso, and M. Kleinfeld. 1965. Bladder Tumors in a Coal Tar Dye Plant. Arch. Environ. Health 11:814-817.

Gorrod, J., R. Carter, and F. Roe. 1968. Induction of Hepatomas by 4-Aminobiphenyl and Three of Its Hydroxylated Derivatives Administered to Newborn Mice. J. Natl. Cancer Inst. 41:403-410.

Graffi, A., F. Hoffman, and M. Schutt. 1967. N-Methyl-N-Nitrosourea As a Strong Topical Carcinogen When Painted on the Skin of Rodents. Nature (London) 214:611.

Grahn, D., and K. F. Hamilton. 1964. Influence of Sex, Environment, and Radiation Factors on Life Shortening and Tumor Incidence in C3H Mice. Radiat. Res. 22:191.

Grasso, P. 1976. Review of Tests for Carcinogenicity and Their Significance to Man. Clin. Toxicol. 9:745-760.

Greenman, D. L., and R. R. Delongchamp. 1979. Variability of Response to Diethylstilbesterol: A Comparison of Inbred with Hybrid Mice. J. Toxicol. and Environ. Health 5:131-143.

Greenman, D., R. Delongchamp, and B. Highman. 1979. Variability of Response to Diethylstilbestrol: A Comparison of Inbred with Hybrid Mice. J. Toxicol. Environ. Health 5:131-143.

Greenwald, P., J. Barlow, P. Nasca, and W. Burnett. 1971. Vaginal Cancer After Maternal Treatment with Synthetic Estrogens. New Engl. J. Med. 285:390-392.

Griesemer, R. A., P. Nettesheim, D. H. Martin, and J. E. Caton, Jr. 1977. Quantitative Exposure of Grafted Rat Tracheas to 7,12-Dimethylbenz(*a*)-anthracene. Cancer Res. 37:1266-1271.

Griswold, D. P., A. E. Casey, E. K. Weisburger, and J. H. Weisburger. 1968. The Carcinogenicity of Multiple Intragastric Doses of Aromatic and Heterocyclic Nitro or Amino Derivatives in Young Sprague-Dawley Rats. Cancer Res. 28:924-933.

Gruenstein, M., D. R. Meranze, and M. B. Shimkin. 1966. Mammary, Sebaceous, and Cutaneous Neoplasms and Leukemia in Male Wistar Rats Receiving Repeated Gastric Instillations of 3-Methylcholanthrene. Cancer Res. 26:2202-2205.

Hayes, W. 1967. Toxicity of Pesticides to Man: Risks from Present Levels. Proc. R. Soc. London Ser. B. 167:101-127.

Herbst, A., L. Ulfelder, and D. Poscanzer. 1971. Adenocarcinoma of the Vagina. Association of Maternal Stilbestrol Therapy with Tumor Appearance in Young Women. New Engl. J. Med. 284:878-881.

Herrold, K. M. 1964*a*. Induction of Olfactory Neuroepithelial Tumors in Syrian Hamsters by Diethylnitrosamine. Cancer 17:114-121.

Herrold, K. 1964*b*. Effect of Route of Administration on the Carcinogenic Action of Diethylnitrosamine. Br. J. Cancer 18:763-767.

Herrold, K. 1969. Aflatoxin Induced Lesions in Syrian Hamsters. Br. J. Cancer 23:655-660.

Herrold, K. M., and L. J. Dunham. 1963. Induction of Tumors in the Syrian Hamster with Diethylnitrosamine (*N*-Nitrosodiethylamine). Cancer Res. 23:773-777.

Heston, W. E. 1958. Mammary Tumors in Agent-Free Mice. Ann. N.Y. Acad. Sci. 71:931-942.

Heston, W. E., and G. Vlahakis. 1966. Factors in the Causation of Spontaneous Heptaomas in Mice. J. Natl. Cancer Inst. 37:839-843.

Heston, W. E., and G. Vlahakis. 1968. C3H-A^{vy} - A High Hepatoma and High Mammary Tumor Strain of Mice. J. Natl. Cancer Inst. 40:1161-1166.

Heston, W. E., G. Vlahakis, and M. K. Deringer. 1960. High Incidence of Spontaneous Hepatomas and the Increase of This Incidence with Urethane in C3H, C3Hf, and C3He Male Mice. J. Natl. Cancer Inst. 24:425-435.

Heuper, W. C., and H. D. Wolf. 1937. Experimental Production of Aniline Tumors of Dogs in the Bladder. Amer. J. Pathol. 13:636-657.

Hiaro, F., T. Fujisawa, E. Tsubura, K. Akamatsu, Y. Yamamura. 1968. Experimental Lung Cancer in Rabbits Induced by Chemical Carcinogens. Gann 59:497-505.

Hoag, W. G. 1963. Spontaneous Cancer in Mice. Ann. N.Y. Acad. Sci. 108:805-831.

Hodge, H., A. Boyce, W. Deichmann, and H. Kraybill. 1967. Toxicology and No-Effect Levels of Aldrin and Dieldrin. Toxicol. Appl. Pharmacol. 10:613-675.

Holland, J. M., D. G. Gosslee, and N. J. Williams. 1979. Epidermal Carcinogenicity of Bis(2,3-epoxycyclopentyl)ether, 2,2-Bis(*p*-glycidyloxyphenyl) propane and m-Phenylenediamine in Male and Female C3H and C57 BL/6 Mice. Cancer Res. 39:1714-1721.

Homburger, F., and J. R. Baker. 1969. Accelerated Carcinogen Testing. Progr. Exp. Tumor Res., Vol. 11. Karger, New York. pp. 384-394.

Homburger, F., and S. S. Hsuch. 1970. Rapid Induction of Subcutaneous Fibrosarcoma by 7,12-Dimethylbenz(*a*)anthracene in an Inbred Line of Syrian Hamsters. Cancer Res. 30:1449-1452.

Homburger, F., S. Hsuch, C. S. Kerr, and A. B. Russfield. 1972. Inherited Susceptibility of Inbred Strains of Syrian Hamsters to Induction of Subcutaneous Sarcomas and Mammary and Gastrointestinal Carcinomas by Subcutaneous and Gastric Administration of Polynuclear Hydrocarbons. Cancer Res. 32:360-366.

Homburger, F., A. B. Russfield. 1970. An Inbred Line of Syrian Hamsters with Frequent Spontaneous Adrenal Tumors. Cancer Res. 30:305-308.

Homburger, F., and A. Tregier. 1960. Modifying Factors in Carcinogenesis. Prog. Exptl. Tumor Res. 1:311.

Howell, J. 1963. The Experimental Production of Vascular Tumors in the Rat. Br. J. Cancer 17:663-671.

Huggins, C., L. C. Grand, and F. P. Brillantes. 1961. Mammary Cancer Induced by a Single Feeding of Polynuclear Hydrocarbons and Its Suppression. Nature 189:204-207.

Huggins, C., L. Grand, and R. Fukunishi. 1964. Aromatic Influences on the Yields of Mammary Cancers Following Administration of 7,12-Dimethylbenz(*a*)anthracene. Proc. Natl. Acad. Sci., Vol. 51. pp. 737-742.

Ihle, J. N., L. O. Arthur, and D. L. Fine. 1976. Autogenous Immunity to Mouse Mammary Tumor Virus in Mouse Strains of High and Low Mammary Tumor Incidence. Cancer Res. 36:2840-2844.

Innes, J.R.M., B. M. Ulland, M. G. Valerio, L. Petrucelli, L. Fishbein, E. R. Hart, A. J. Pallotta, R. R. Bates, H. L. Falk, T. J. Gart, M. Klein, I. Mitchell, and J. Peters. 1969. Bioassay of Pesticides and Industrial Chemicals for Tumorigenicity in Mice. J. Natl. Cancer Inst. 42:1101-1114.

Ivankovic, S. 1973. Experimental Prenatal Carcinogenesis. In: Transplacental Carcinogenesis, L. Tomatis and U. Mohr, eds. Scientific Publication No. 4, International Agency for Research on Cancer, Lyon, France. pp. 92-99.

Jalanko, H., I. Virtanen, E. Engvall, and E. Ruoslahti. 1978. Early Increase of Serum Alpha-Fetoprotein in Spontaneous Hepatocarcinogenesis in Mice. Int. J. Cancer 21(4):453-459.

Jull, J. W. 1951. The Induction of Tumors of the Bladder Epithelium in Mice by the Direct Application of a Carcinogen. Br. J. Cancer 5:328-330.

Jungherr, E. 1963. Spontaneous Cancer in Zoo and Laboratory Animals. Part II. Tumors and Tumor-Like Conditions in Monkeys. Ann. N.Y. Acad. Sci. 108:777-792.

Kaplan, A., and H. Sherman. 1977. Toxicity Studies with Methyl-*N*-[(Methyllamine-carbonyl)oxyl]-ethanimidothioate. Toxicol. Appl. Pharmacol. 40:1-17.

Kaye, A., and N. Trainin. 1966. Urethan Carcinogenesis and Nucleic Acid Metabolism: Factors Influencing Lung Adenoma Induction. Cancer Res. 26:2206-2212.

Kelly, M., and R. O'Gara. 1961. Induction of Tumors in Newborn Mice with Dibenz(*a*)anthracene and 3-Methylcholanthrene. J. Natl. Cancer Inst. 26:651-679.

Kelly, M. G., R. W. O'Gara, R. H. Adamson, K. Gadekar, C. C. Botkin, W. H. Reese, Jr., and W. T. Kerber. 1966. Induction of Hepatic Cell Carcinomas in Monkeys with *N*-Nitrosodiethylamine. J. Natl. Cancer Inst. 36:323-351.

Ketkar, M., G. Reznik, P. Schneider, and U. Mohr. 1978. Investigations on the Carcinogenic Burden by Air Pollution in Man. Intratracheal Instillation Studies with Benzo(*a*)pyrene in Bovine Serum Albumin in Syrian Hamsters. Cancer Lett. 4:235-239.

Klein, M. 1951. The Action of Croton Oil in the Induction of Sarcomas in Mice. J. Natl. Cancer Inst. 11:843-848.

Klein, M. 1952. The Action of Methylcholanthrene, Croton Oil, and 1,2-Benzanthracene in the Induction of Skin Tumors in Strain DBA Mice. J. Natl. Cancer Inst. 12:735-742.

Klein, M. 1953. Effects of Croton Oil on Induction of Tumors by 1,2-Benzanthracene, Deoxycholic Acid, or Low Doses of 20-MCA in Mice. J. Natl. Cancer Inst. 13:333.

Klein, M. 1963. Susceptibility of Strain BLoAF$_1$/J Hybrid Infant Mice to Tumorigenesis with 1,2-Benzanthracene, Deoxycholic Acid, and 3-Methylcholanthrene. Cancer Res. 23(6):1701-1707.

Krasovskii, G. 1976. Extrapolation of Experimental Data from Animals to Man. Environ. Health Perspect. 13:51-58.

Kroes, R., G. M. Williams, and J. H. Weisburger. 1973. Early Appearance of Serum *a*-Fetoprotein as a Function of Dosage of Various Hepatocarcinogens. Cancer Res. 33:613-617.

Kuschner, M., S. Laskin, E. Cristofano, and N. Nelson. 1957. Experimental Carcinoma of the Lung. In: Proceedings of the Third National Cancer Conference. Lippincott, Philadelphia. pp. 485-495.

Laishes, B., and E. Farber. 1978. Transfer of Viable Putative Preneoplastic Hepatocytes to the Livers of Syngenic Host Rats. J. Natl. Cancer Inst. 61(2):507-512.

Laishes, B. A., L. Fink, and B. I. Carr. 1980. A Liver Colony Assay for a New Hepatocyte Phenotype as a Step Towards Purifying New Cellular Phenotypes That Arise During Hepatocarcinogenesis. Ann. N.Y. Acad. Sci. (in press).

Lawrence, W. 1976. Systemic Toxicity. Acute vs. Subchronic and Chronic. Drug. Cosmet. Ind. November 1976:40-114.

Laws, J. O., and A. Flaks. 1966. Pulmonary Adenomata Induced by Carcinogen Treatment in Organ Culture. Br. J. Cancer 20:550-554.

Lee, C-C., J. Q. Russell, and J. L. Minor. 1978. Oral Toxicity of Ferric Dimethyl-Dithiocarbamate (Ferbam) and Tetramethylthiuram Disulfide (Thiram) in Rodents. J. Toxicol. Environ. Health 4:93-106.

Lee, K., B. Toth, and P. Shubik. 1963. Carcinogenic Response of the Syrian Golden Hamster Treated at Birth with 7,12-Dimethylbenz(*a*)-anthracene. Proc. Soc. Exptl. Biol. Med. 114:579-582.

Levy, B. M. 1963. Induction of Fibrosarcoma in the Primate, *Tamarinus nigricollis*. Nature 200:182-183.

Liebelt, R., A. Liebelt, and M. Lane. 1964. Hormonal Influences on Urethan Carcinogenesis in C3H/f Mice. Cancer Res. 24:1869-1879.

Liebelt, R., R. Yoshida, and G. Gray. 1961. Enhancement of Liver Tumorigenesis in Zb Mice Injected with Urethan at Newborn Age. Proc. Assoc. Cancer Res. 3:245.

Lijinsky, W., A. Ferrero, R. Montesano, and C.E.M. Wenyon. 1970. Tumorigenicity of Cyclic Nitrosamines in Syrian Golden Hamsters. Z. Krebsforsch. 74:185-189.

Lijinsky, W., K. Lee, Y. Tomatis, and W. H. Butler. 1967. Nitrosoazetidine — a Potent Carcinogen of Low Toxicity. Naturwissenschaften 54:518.

Lijinsky, W., and H. W. Taylor. 1978. Relative Carcinogenic Effectiveness of Derivatives of Nitrosodiethylamine in Rats. Cancer Res. 38: 2391-2394.

Loomis, T. 1974. Essentials of Toxicology, 2nd ed., Lea and Febiger, Philadelphia. pp. 189-191.

Luz, A. 1977. The Range of Incidence of Spontaneous Neoplastic and Non-Neoplastic Lesions of the Laboratory Mouse. Z. Versuchstierk. 19(6): 342-343.

Maekawa, A., and S. Odashima. 1975. Spontaneous Tumors in ACI/N Rats. J. Natl. Cancer Inst. 55:1437-1445.

Magee, P. 1970. Tests for Carcinogenic Potential. In: Methods in Toxicology, G. Paget, ed. F. A. Davis Company, Philadelphia. pp. 158-196.

McNamara, B. 1976. Concepts in Health Evaluation of Commercial and Industrial Chemicals. In: Advances in Modern Toxicology, Part 1, Vol. 1, M. Mehlman, R. Shapiro, and H. Blumenthal, eds. John Wiley and Sons, New York. pp. 61-140.

Milievskaja, I. L., and N. S. Kiseleva. 1976. Comparative Study of the Carcinogenic Activities of NAS and Some Chemical Carcinogens When Introduced into the Buccal Pouch of the Syrian Hamster. Bull. World Health Organ. 54:607-614.

Miller, E. C., J. A. Miller, and M. Enomota. 1964. The Comparative Carcinogenicities of 2-Acetylaminofluorene and Its *N*-Hydroxy Metabolite in Mice, Hamsters and Guinea Pigs. Cancer Res. 24:2018-2031.

Ministry of Health and Welfare Canada. 1975. The Testing of Chemicals for Carcinogenicity, Mutagenicity, and Teratogenicity. Ministry of Health and Welfare, Ottawa. 183 pp.

Mohr, U. 1973. Effects of Diethylnitrosamine on Fetal and Suckling Syrian Golden Hamsters. In: Transplacental Carcinogenesis, L. Tomatis and U. Mohr, eds. Scientific Publication No. 4, International Agency for Research on Cancer, Lyon, France. pp. 65-70.

Mohr, U., G. Reznik, and P. Pour. 1977. Carcinogenic Effects of Diisopropanolnitrosamine in Sprague-Dawley Rats. J. Natl. Cancer Inst. 58:361-366.

Morris, H. P., and W. H. Eyestone. 1953. Tumors of the Liver and Urinary Bladder of Dogs After Ingestion of 2-Acetylaminofluorene. J. Natl. Cancer Inst. 13:1139-1165.

Morris, H. P., and H. I. Firminger. 1956. Influence of Sex and Sex Hormones on Development of Hepatomas and Other Hepatic Lesions in Strain AXC Rats Ingesting 2-Diacetylaminofluorene. J. Natl. Cancer Inst. 16:927-949.

Mulay, A. S., and R. W. O'Gara. 1959. Incidence of Liver Tumors in Male and Female Rats Fed Carcinogenic Azo Dyes. Proc. Soc. Exptl. Biol. Med. 100:320-322.

Munro, I. 1977. Considerations in Chronic Toxicity Testing: The Chemical, the Dose, the Design. J. Environ. Pathol. Toxicol. 1:183-197.

Nagasawa, H., R. Yanai, and I. Azuma. 1978. Suppression by *Nocardia rubra* Cell Wall Skeleton of Mammary DNA Synthesis, Plasma Prolactin Level, and Spontaneous Tumorigenesis in Mice. Cancer Res. 38:2160-2162.

National Academy of Sciences. 1975. Principles for Evaluating Chemicals in the Environment. National Academy of Sciences, Washington, D.C. pp. 115-153.

National Academy of Sciences. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. National Academy of Sciences, Washington, D.C. 130 pp.

National Institutes of Health. 1968-1973. Survey of Compounds Which Have Been Tested for Carcinogen Activity. Public Health Service Publication No. 149, Government Printing Office, Washington, D.C.

Neiman, J. M. 1968. The Sensitizing Carcinogenic Effect of Small Doses of Carcinogen. Europ. J. Cancer 4:537-545.

Nelson, A. A., O. G. Fitzhugh, and H. O. Calvery. 1943. Liver Tumors Following Cirrhosis Caused by Selenium in Rats. Cancer Res. 3:230-236.

Nelson, A. A., and G. Woodard. 1953. Tumors of the Urinary Bladder, Gall Bladder and Liver in Dogs Fed *O*-Aminoazotoluene or *p*-Dimethylaminoazobenzene. J. Natl. Cancer Inst. 13:1497-1509.

Nettesheim, P., and R. A. Griesemer. 1978. Experimental Models for Studies of Respiratory Tract Carcinogenesis. In: Pathogenesis and Therapy of Lung Cancer, Lung Biology in Health and Disease 10, C. Harris, ed. New York. pp. 75-188.

Nettesheim, P., and A. S. Hammons. 1971. Induction of Squamous Cell Carcinoma in the Respiratory Tract of Mice. J. Natl. Cancer Inst. 47:697-701.

Newberne, P. M., and A. Rogers. 1972. Vitamin A, Liver, Colon Carcinoma in Rats Fed Low Levels of Aflatoxin. Toxicol. Appl. Pharmacol. 22:280.

Nishizuka, Y., K. Nakakuki, and T. Sakakura. 1964. Induction of Pulmonary Tumors and Leukemia by a Single Injection of 4-Nitroquinoline 1-Oxide to Newborn and Infant Mice. Gann 55:495-508.

Noble, R., B. C. Hochachka, and D. King. 1975. Spontaneous and Estrogen-Produced Tumors in Nb Rats and Their Behavior After Transplantation. Cancer Res. 35:766-780.

Noyes, W. F. 1968. Carcinogen Induced Sarcoma in the Primitive Primate, *Tupaia glis*. Proc. Soc. Exp. Biol. Med. 127:594-596.

Noyes, W. F. 1969. Carcinogen-Induced Neoplasia with Metastasis in a South American Primate, *Saquinus oedipus*. Proc. Soc. Expt. Biol. Med. 131:223-225.

O'Gara, R. W., and M. G. Kelly. 1965. Pulmonary Arteriolitis in Infant Monkeys Produced by Subcutaneous Injections of Polycyclic Hydrocarbons. Arch. Path. (Chicago) 79:475-483.

Oppenheimer, B. S., E. T. Oppenheimer, and A. P. Stout. 1948. Sarcomas Induced by Rats by Implanting Cellophane. Proc. Soc. Expt. Biol. Med. 67:33-34.

Outzen, H. C., R. Custer, R. Phillips, G. J. Eaton, and R. T. Prehn. 1975. Spontaneous and Induced Tumor Incidence in Germ Free Nude Rats. J. Reticuloendothel. Soc. 17(1):1-9.

Page, N. P. 1977*a*. Concepts of a Bioassay Program in Environmental Carcinogenesis. In: Advances in Modern Toxicology, Vol. 3: Environmental Cancer, H. Kraybill and M. Mehlman, eds. Hemisphere Publishing Corp., Washington, D.C. pp. 87-171.

Page, N. 1977*b*. Chronic Toxicity and Carcinogenicity Guidelines. J. Environ. Pathol. Toxicol. 1:161-182.

Peacock, P. M. 1962. A Short-Term Test for Carcinogenicity (The Effects of Certain Closely-Related Polycyclic Aromatic Hydrocarbons on Embryo Tissue Homografts in BALB/c Strain Mice). Br. J. Cancer 16:701-706.

Peacock, P. M., and E. Dick. 1963. A Short-Term Test for Carcinogenicity (Mouse Embryo Tissue Homografts in BALB/c Strain Mice). Br. J. Cancer 17:59-61.

Peck, H. 1968. An Appraisal of Drug Safety Evaluation in Animals and the Extrapolation of Results to Man. In: Importance of Fundamental Principles in Drug Evaluation, D. Tedeschi and R. Tedeschi, eds. Raven Press, New York. pp. 449-471.

Peck, H. 1974. Design of Experiments to Detect Carcinogenic Effects of Drugs. In: Carcinogenesis Testing of Chemicals, L. Goldberg, ed. The Chemical Rubber Company, Cleveland, Ohio. pp. 1-14.

Peraino, C., R.J.M. Fry, and E. Staffeldt. 1973. Enhancement of Spontaneous Hepatic Tumorigenesis in C3H Mice by Dietary Phenobarbital (brief communication). J. Natl. Cancer Inst. 51:1349-1350.

Percy, D. H., and A. M. Jonas. 1971. Incidence of Spontaneous Tumors in CD^{R}-1 H*a*M/ICR Mice. J. Natl. Cancer Inst. 46:1045-1053.

Petzold, G. L. and J. A. Swenberg. 1978. Detection of DNA Damage Induced *In Vivo* Following Exposure of Rats to Carcinogens. Cancer Res. 38: 1589-1594.

Pietra, G., H. Rappaport, and P. Shubik. 1961. The Effects of Carcinogenic Chemicals in Newborn Mice. Cancer 14:308-317.

Pietra, G., K. Spencer, and P. Shubik. 1959. Response of Newly Born Mice to a Chemical Carcinogen. Nature 183:1689.

Pollard, M., and M. Kajima. 1970. Lesions in Aged Germ Free Wistar Rats. Amer. J. Pathol. 61:25-32.

Pott, F., A. Brockhaus, and F. Huth. 1973. Tests on the Production of Tumors in Animal Experiments with Polycyclic Aromatic Hydrocarbons. Zbl. Bakt. Hyg., I. Abt. Orig. B 157:34-43.

Pott, F., R. Tomingas, and J. Misfeld. 1977. Tumors in Mice After Subcutaneous Injection of Automobile Exhaust Condensates. In: Air Pollution and Cancer in Man, Mohr, Sahmahl, and Tomatis, eds. International Agency for Research on Cancer. pp. 79-87.

Pour, P., F. W. Kruger, and J. Althoff. 1974. Cancer of the Pancreas Induced in the Syrian Golden Hamster. Am. J. Pathol. 76:349-358.

Pour, P., F. W. Kruger, and J. Althoff. 1975. Effect of Beta-oxidized Nitrosamines on Syrian Hamsters. III. 2,2-Dihydroxy-di-*n*-propylnitrosamine. J. Natl. Cancer Inst. 54:141-145.

Pour, P., N. Kmoch, E. Greiser, U. Mohr, J. Althoff, and A. Cardesa. 1976. Spontaneous Tumors and Common Diseases in Two Colonies of Syrian Hamsters. J. Natl. Cancer Inst. 56(6):931-935.

Rall, D. 1969. Difficulties in Extrapolating the Results of Toxicity Studies in Laboratory Animals to Man. Environ. Res. 2:360-367.

Reeves, A. L., H. E. Puro, and R. G. Smith. 1974. Inhalation Carcinogenesis from Various Forms of Asbestos. Environ. Res. 8:178-202.

Reuber, M. D. 1976. Effect of Age and Sex on Lesions of the Esophagus in Buffalo Strain Rats Ingesting Diethylnitrosamine. Exp. Cell Biol. 44:65-72.

Reuber, M. D., and F. L. Glover. 1967. Hyperplastic and Early Neoplastic Lesions of the Liver in Buffalo Strain Rats of Various Ages Given Subcutaneous Carbon Tetrachloride. J. Natl. Cancer Inst. 38:891-899.

Reznik, G. 1975. The Carcinogenic Effect of (DMN) on the Chinese Hamster (*Cricetulus Griseus*). Cancer Lett. 1:25-28.

Rice, J. 1976. Carcinogenesis: A Late Effect of Irreversible Toxic Damage During Development. Environ. Health Perspect. 18:133-139.

Rigdon, R. H., M. C. Benge, H. Kirchoff, J. Mack, and J. Neal. 1969. Leukemia in Mice Fed Benzo(*a*)pyrene: A Clinical, Pathologic and Hematologic Study. Tex. Rep. Biol. Med. 27:803-820.

Rigdon, R. H., and J. Neal. 1966. Gastric Carcinomas and Pulmonary Adenomas in Mice Fed Benzo(*a*)pyrene. Tex. Rep. Biol. Med. 24:195-207.

Riley, V. 1975. Mouse Mammary Tumors: Alteration of Incidence as Apparent Function of Stress. Science 189:465-467.

Roe, F. 1975. Neonatal Induction of Hepatic and Other Tumors. In: Mouse Hepatic Neoplasia, W. Butler and P. M. Newbern, eds. Elsevier Scientific Publishing Co., New York. Chapter 7.

Roe, F., R. Carter, and S. Adamthwaite. 1969. Induction of Liver and Lung Tumors in Mice by 6-Aminochrysene Administered During the First Three Days of Life. Nature (London) 221:1063-1064.

Roe, F., B. Mitchley, and M. Walters. 1963. Tests for Carcinogenesis Using Newborn Mice: 1,2-Benzanthracene, 2-Naphthylamine, 2-Naphthylhydroxylamine and Ethyl Methane Sulphonate. Br. J. Cancer 17:255-260.

Roe, F., K. Rowsen, and M. Salaman. 1961. Tumours of Many Sites Induced by Injection of Chemical Carcinogenesis into Newborn Mice, A Sensitive Test for Carcinogenesis: The Implication for Certain Immunological Theories. Br. J. Cancer 15:515-530.

Rosenkrantz, H., R. Sprague, R. Fleischman, and M. Braude. 1975. Oral Δ^9-Tetrahydrocannabinol Toxicity in Rats Treated for Periods up to 6 Months. Toxicol. Appl. Pharmacol. 32:399-417.

Rygaard, J., and C. O. Polvson. 1974. The Mouse Mutant Nude Does Not Develop Spontaneous Tumors. Acta. Pathol. Microbiol. Scand. 82: 99-106.

Sabine, J. R., B. J. Horton, and M. B. Wicks. 1973. Spontaneous Tumors in C3H-A^{vy} and C3H-A^{vy}fB Mice: High Incidence in the United States and Low Incidence in Australia. J. Natl. Cancer Inst. 50:1237-1242.

Saffiotti, U., F. Cefis, R. Montesano, and A. R. Sellakumar. 1966. Induction of Bladder Cancer in Hamsters Fed Aromatic Amines. Ind. Med. Surg. 35:564.

Sass, B., L. S. Rabstein, R. Madison, R. M. Nims, R. L. Peters, and G. J. Kelloff. 1975. Incidence of Spontaneous Neoplasms in F344 Rats Throughout the Natural Life-Span. J. Natl. Cancer Inst. 54:1449-1456.

Schepers, G.W.H. 1971. Lung Tumors of Primates and Rodents. Part II. Inhalation Experiments. Ind. Med. May:23-31.

Schmähl, D., and H. Osswald. 1967. Carcinogenesis in Different Animal Species by Diethylnitrosamine. Experentia 23:497-498.

Schrauzer, G. N., D. A. White, J. E. McGinness, and C. J. Schneider. 1978. Arsenic and Cancer: Effects of Joint Administration of Arsenite and Selenite on the Genesis of Mammary Adenocarcinoma in Inbred Female C_3H/St Mice. Bioinorg. Chem. 9:245-253.

Schreiber, H., K. Schreiber, and D. H. Martin. 1975. Experimental Tumor Induction in a Circumscribed Area of the Hamster Trachea: Correlation of Histology and Exfoliative Cytology. J. Natl. Cancer Inst. 54:187-197.

Shimkin, M. B. 1940. Induced Pulmonary Tumors in Mice. II. Reaction of Lungs of Strain A Mice to Carcinogenic Hydrocarbons. Arch. Path. 29:239-255.

Shimkin, M. B., and G. D. Stoner. 1975. Lung Tumors in Mice: Application to Carcinogenesis Bioassay. Adv. Cancer Res. 21:1-58.

Shirasu, Y. 1965. Comparative Carcinogenicity of 4-Nitroquinoline 1-Oxide and 4-Hydroxyaminoquinoline 1-Oxide in Three Strains of Mice. Proc. Soc. Exptl. Biol. Med. 118:812-814.

Shubik, P. 1950. The Growth Potentialities of Induced Skin Tumors in Mice. The Effects of Different Methods of Chemical Carcinogenesis. Cancer Res. 10:713-717.

Shubik, P. 1972. The Use of the Syrian Golden Hamster in Chronic Toxicity Testing. Prog. Exp. Tumor Res. 16:176-184.

Silverstone, H., and A. Tannenbaum. 1951. Proportion of Dietary Protein and the Formation of Spontaneous Hepatomas in the Mouse. Cancer Res. 11:442-446.

Snell, K. C. 1965. Spontaneous Lesions of the Rat. In: The Pathology of Laboratory Animals, W. E. Ribelin and J. R. McCoy, eds. Charles C. Thomas, Publisher, Springfield, Illinois.

Solt, D. B., A. Medline, and E. Farber. 1977. Rapid Emergence of Carcinogen-Induced Hyperplastic Lesions in a New Model for the Sequential Analysis of Liver Carcinogenesis. Am. J. Path. 88:595-618.

Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. NCI-CG-TR-1, February. Bethesda, Maryland.

Spatz, M., and G. Laqueur. 1967. Transplacental Induction of Tumors in Sprague-Dawley Rats with Crude Cyad Material. J. Natl. Cancer Inst. 38:233-245.

Spitz, S., W. H. Maguigan, and K. Dobriner. 1950. The Carcinogenic Action of Benzidine. Cancer 3:789-804.

Steinmuller, D., L. A. Dillingham, and R. T. Prehn. 1969. Lack of Carcinogenic Activity of 3-Methylcholanthrene in the Squirrel Monkey. J. Natl. Cancer Inst. 43:1175-1180.

Stevens, L. C. 1973. A New Inbred Subline of Mice (129/terSv) with a High Incidence of Spontaneous Congenital Testicular Teratomas. J. Natl. Cancer Inst. 50:235-242.

Stevenson, D. 1979. Current Problems in the Choice of Animals for Toxicity Testing. J. Toxicol. Environ. Health 5:9-15.

Stoltz, D. R., L. A. Poirer, C. C. Irving, H. F. Stich, J. H. Weisburger, and H. C. Frice. 1974. Evaluation of Short-Term Tests for Carcinogenicity. Tox. Appl. Pharmacol. 29:157-180.

Street, A. 1970. Biochemical Tests in Toxicology. In: Methods in Toxicology, G. Paget, ed. F. A. Davis Company, Philadelphia. pp. 313-337.

Strong, L. C., and H. Matsunaga. 1975. An Inverse Correlation (Dose-Response) Between 5-Methyl-Cyiidine and the Fate of Spontaneous Tumors in Mice. Cytobios 12:13-18.

Sugiura, K., W. E. Smith, and D. A. Sunderland. 1956. Experimental Production of Carcinoma in Rhesus Monkeys. Cancer Res. 16:951.

Sumi, N., D. Stavrou, H. Frohberg, and G. Jochmann. 1976. The Incidence of Spontaneous Tumors of the Central Nervous System of Wistar Rats. Arch. Toxicol. 35:1-13.

Sunderman, F. W., Jr. 1971. Metal Carcinogenesis in Experimental Animals. Food Cosmet. Toxicol. 9:105-120.

Svenberg, J., A. Kostner, W. Wechsler, and R. Denlinger. 1972. Quantitative Aspects of Transplacental Tumor Induction with Ethylnitrosourea in Rats. Cancer Res. 32:2656-2660.

Swenberg, J. A., G. L. Petzold, and P. R. Harbach. 1976. In Vitro DNA Damage/Alkaline Elution Assay for Predicting Carcinogenic Potential. Biochem. Biophys. Res. Comm. 72:732-738.

Syndor, K. L. 1973. A Preliminary Investigation of the Susceptibility to Sarcoma Induction by Low Doses of B(*a*)P in Nine Strains of Inbred Male Rats. Proceedings Tobacco and Health Workshop Conf., 4th University of Kentucky Tobacco and Health Research Institute.

Syndor, K. L., O. Butenandt, F. P. Brillantes, and C. Huggins. 1962. Race - Strain Factor Related to Hydrocarbon-Induced Mammary Cancer in Rats. J. Natl. Cancer Inst. 29:805-814.

Tannenbaum, A., and H. Silverstone. 1949. The Influence of the Degree of Caloric Restriction on the Formation of Skin Tumors and Hepatomas in Mice. Cancer Res. 9:724-727.

Tatematsu, M., T. Shirai, H. Tsuda, Y. Miyata, Y. Shinohara, and N. Ito. 1977. Rapid Production of Hyperplastic Liver Nodules in Rats Treated with Carcinogenic Chemicals: A New Approach for an *In Vivo* Short-Term Screening Test for Hepatocarcinogens. Gann 68:499-507.

Terracini, B., P. N. Magee, and J. M. Barnes. 1967. Hepatic Pathology in Rats on Low Dietary Levels of Dimethylnitrosamine. Br. J. Cancer 21:559-565.

Terracini, B., G. Palestro, M. Gigliardi, and R. Montesano. 1966. Carcinogenicity of Dimethylnitrosamine in Swiss Mice. Br. J. Cancer 20: 871-876.

Terracini, B., M. C. Testa, J. R. Cabral, and N. Day. 1973. The Effects of Long-Term Feeding of DDT to BALB/c Mice. Int. J. Cancer 11:747-764.

Thiery, M., and M. van Gijsegem. 1965. Species and Strain Differences in the Susceptibility of the Cervico-Vaginal Squamous Epithel. to 3,4-Benzo(*a*)pyrene. Br. J. Cancer 19:418-429.

Tomatis, L., C. Agthe, H. Bartsch, J. Huff, R. Montesano, R. Saracci, E. Walker, and J. Wilbourn. 1978. Evaluation of the Carcinogenicty of Chemicals: A Review of the Monograph Program of the International Agency for Research on Cancer (1971 to 1977). Cancer Res. 38:877-885.

Tomatis, L., G. Della Porta, and P. Shubik. 1961. Urinary Bladder and Liver Cell Tumors Induced in Hamsters with *o*-Aminoazotoluene. Cancer Res. 21:1513-1517.

Tomatis, L., and V. Turusov. 1975. Studies on the Carcinogenicity of DDT. Gann Monograph on Cancer Res. 17:219-241.

Tomatis, L., V. Turusov, D. Guibbert, B. Duperray, C. Malaveille, and H. Pacheco. 1971. Transplacental Carcinogenic Effect of 3-Methylcholanthrene in Mice and Its Quantitation in Fetal Tissues. J. Natl. Cancer Inst. 47:645-651.

Toth, B. 1968. A Critical Review of Experiments in Chemical Carcinogenesis Using Newborn Animals. Cancer Res. 28:727-738.

Toth, B., P. Magee, and P. Shubik. 1964. Carcinogenesis Study with Dimethylnitrosamine Administered Orally to Adult and Subcutaneously to Newborn BALB/c Mice. Cancer Res. 24:1712-1721.

Toth, B., H. Rappaport, and P. Shubik. 1963. Influence of Dose and Age on the Induction of Malignant Lymphomas and Other Tumors by 7,12-Dimethylbenz(*a*)anthracene in Swiss Mice. J. Natl. Cancer Inst. 30:723-741.

Toth, B., and P. Shubik. 1963. Carcinogenesis in Lewis Rats Injected at Birth with 7,12-Dimethylbenz(*a*)anthracene. Br. J. Cancer 17:540-545.

Turner, F. C. 1941. Sarcomas at the Site of Subcutaneously Implanted Bakelite Discs in Rats. J. Natl. Cancer Inst. 2:81.

Turusov, V., L. Tomatis, D. Guibbert, B. Duperray, and H. Pacheco. 1973. The Effect of Prenatal Exposure of Mice to Methylcholanthrene Combined with the Neonatal Administration of Diethylnitrosamine. In: Transplacental Carcinogenesis, L. Tomatis and U. Mohr, eds. Scientific Publication No. 4, International Agency for Research on Cancer, Lyon, France. pp. 84-91.

U.S. Food and Drug Administration. 1971. Advisory Committee on Protocols for Safety Evaluation, Panel on Carcinogenesis Report on Cancer Testing in the Safety Evaluation of Food Additives and Pesticides. Toxicol. Appl. Pharmacol. 20:419-438.

Verschuuren, H., R., Kroes, and G. Van Esch. 1973. Toxicity Studies on Tetrasul. I. Acute, Long-Term, and Reproduction Studies. Toxicology 1:63-78.

Vesselinovitch, S. 1973. Comparative Studies on Perinatal Carcinogenesis. In: Transplacental Carcinogenesis, L. Tomatis and U. Mohr, eds. Scientific Publication No. 4, International Agency for Research on Cancer, Lyon, France. pp. 14-22.

Vesselinovitch, S. D., A. P. Kyriazis, N. Mihailovich, and K.V.N. Rao. 1975. Conditions Modifying Development of Tumors in Mice at Various Sites by Benzo(*a*)pyrene. Cancer Res. 35:3948-2953.

Vesselinovitch, S., and N. Mihailovich. 1966. Significance of Newborn Age and Dose of Urethan in Leukemogenesis. Cancer Res. 26:1633-1637.

Vesselinovitch, S, N. Mihailovitch, and L. Itze. 1970. Comparative Studies on the Kinetics of the Neoplastic Competence in Mice. Cancer Res. 30:2548-2551.

Vesselinovitch, S., N. Mihailovich, G. Wogan, L. Lombard, and K. Rao. 1972. Aflatoxin B_1, a Hepatocarcinogen in the Infant Mouse. Cancer Res. 32:2289-2291.

Vesselinovitch, S., K. Rao, and N. Mihailovich. 1975. Factors Modulating Benzidine Carcinogenicity Bioassay. Cancer Res. 35:2814-2819.

Vlahakis, G., W. E. Heston, and G. H. Smith. 1970. Strain $C3H\text{-}A^{vy}fB$ Mice: Ninety Percent Incidence of Mammary Tumors Transmitted by Either Parent. Science 170:185-187.

Walpole, A. L., M.H.C. Williams, and D. C. Roberts. 1952. The Carcinogenic Action of 4-Aminodiphenyl and 3:2'Dimethyl-4-Aminodiphenyl. Br. J. Ind. Med. 9:255-263.

Walpole, A. L., M.H.C. Williams, and D. C. Roberts. 1954. Tumors of the Urinary Bladder in Dogs After Ingestion of 4-Aminodiphenyl. Br. J. Ind. Med. 11:105-109.

Watabe, H. 1971. Early Appearance of Embryonic α-Globulin in Rat Serum During Carcinogenesis with 4-Dimethylaminobenzene. Cancer Res. 31: 1192-1194.

Watanabe, P., J. Young, and P. Gehring. 1977. The Importance of Non-Linear (Dose-Dependent) Pharmacokinetics in Hazard Assessment. J. Environ. Pathol. Toxicol. 1:147-159.

Weil, C. 1962. Applications of Methods of Statistical Analysis to Efficient Repeated-Dose Toxicological Tests: 1. General Considerations and Problems Involved. Sex Differences in Rat Liver and Kidney Weights. Toxicol. Appl. Pharmacol. 4:561-571.

Weil, C. 1972. Guidelines for Experiments to Predict the Degree of Safety of a Material for Man. Toxicol. Appl. Pharmacol. 21:194-199.

Weil, C. 1973. Experimental Design and Interpretation of Data from Prolonged Toxicity Studies. Pharmacology and the Future of Man. Proc. 5th Int. Congr. Pharmacol., San Francisco, 1972, Vol. 2: Toxicological Problems, Karger, New York. pp. 4-12.

Weil, C., and C. Carpenter. 1969. Abnormal Values in Control Groups During Repeated-Dose Toxicologic Studies. Toxicol. Appl. Pharmacol. 14:335-339.

Weil, C., and D. McCollister. 1963. Safety Evaluation of Chemicals. Relationships Between Short- and Long-Term Feeding Studies in Designing and Effective Toxicity Test. Agric. Food Chem. 11:486-491.

Weil, C., M. Woodside, H. Smyth, Jr., and C. Carpenter. 1971. Results of Feeding Propylene Glycol in the Diet of Dogs for Two Years. Food Cosmet. Toxicol. 9:479-490.

Weisburger, E. K. 1977. Carcinogenicity Studies on Halogenated Hydrocarbons. Environ. Health Perspect. 21:7-16.

Weisburger, J. H. 1976. Bioassays and Tests for Chemical Carcinogens. In: Chemical Carcinogens, American Chemical Society, Monograph 173, C. E. Searle, ed. Washington, D.C. pp. 1-23.

Weisburger, J., M. Klein, E. Weisburger, R. Glass, G. Woodard, and M. Gronin. 1970. Comparison of the Effect of the Carcinogen *N*-Hydroxyl-*N*-2-Fluorenylacetamide in Infant and Weanling Rats. J. Natl. Cancer Inst. 45:29-35.

Weisburger, J. H., and E. K. Weisburger. 1967. Tests for Chemical Carcinogens. From: Methods in Cancer Res., Vol. I., H. Busch, ed. Academic Press, New York. pp. 307-398.

Welsch, C. W. 1976. Interaction of Estrogen and Prolactin in Spontaneous Mammary Tumorigenesis of the Mouse. J. Toxicol. Environ. Health Suppl. 1:161-175.

Wigley, C. B., J. Amos, and P. Brookes. 1978. Different Tumours Induced by Benzo(*a*)pyrene and Its 7,8-Dihydrodiol Injected into Adult Mouse Salivary Gland. Br. J. Cancer 37:657-661.

Winstead, J. 1978. What Kinds of Tests Are Prerequisite to Labeling a Compound as Toxic? Int. J. Occup. Health Saf. 47(3):26-28.

Wislocki, P. G., R. L. Chang, A. W. Wood, W. Levin, H. Yagi, O. Hernandez, H. D. Mah, P. M. Dansette, D. M. Jerina, and A. H. Conney. 1977. High Carcinogenicity of 2-Hydroxybenzo(*a*)pyrene on Mouse Skin. Cancer Res. 37:2608-2611.

Wogan, G. N., and R. C. Shank. 1971. Toxicity and Carcinogenicity of Aflatoxins. In: Advances in Environmental Science and Technology, J. N. Pitts and R. L. Metcalf, eds., John Wiley & Sons, Inc., New York. pp. 321-350.

Womack, J. 1979. Genetic Constitution and Response to Toxic Chemicals — An Overview. J. Toxicol. Environ. Health 5:49-51.

World Health Organization. 1978. Principles and Methods for Evaluating the Toxicity of Chemicals. Part 1. Geneva. 274 pp.

Yoshida, T. 1932. Uber die Experimantelle Erzeugung von Hepatom durch die Futterung mit o-Amido-azotoluol. Proc. Imp. Acad. Japan 8:464-467.

Zabezhinskii, M. A. 1970. Effectiveness of Inhalation as a Method of Administration of Atomized Carcinogens. Bull. Exptl. Biol. and Med. 69:68-70.

Zackheim, H. S. 1964. Comparative Cutaneous Carcinogenesis in the Rat. Oncologia 17:236-246.

Zbinden, G. 1973. Formal Toxicology. In: Progress in Toxicology, Special Topics, Vol. 1. Springer-Verlag, New York. pp. 4-27.

Zoller, M., S. Matzku, and K. Goerttler. 1978. High Incidence of Spontaneous Transplantable Tumours in BDX Rats. Brit. J. Cancer 37:61-66.

BIBLIOGRAPHY FOR SECTION 5

Abrahms, W., G. Zbinden, and R. Bagdon. 1965. Investigative Methods in Clinical Toxicology. J. New Drugs 5:199-207.

Adamson, R. H., R. W. Cooper, and R. W. O'Gara. 1970. Carcinogen-Induced Tumors in Primitive Primates. J. Natl. Cancer Inst. 45:555-560.

Adamson, R. H., P. Correa, and D. W. Dalgard. 1973. Occurrence of a Primary Liver Carcinoma in a Rhesus Monkey Fed Aflatoxin B_1. J. Natl. Cancer Inst. 50(2):549-553.

Agthe, C., H. Garcia, P. Shubik, L. Tomatis, and E. Wenyon. 1970. Study of the Potential Carcinogenicity of DDT in the Syrian Golden Hamster. Proc. Soc. Exp. Biol. Med. 134:113-116.

Akamatsu, Y., and B. Barton. 1974. Neoplasms and Amyloidosis in Strains of Mice Treated with 3-Methylcholanthrene. J. Natl. Cancer Inst. 52:377-385.

Alfred, L. J., A. Globerson, Y. Berwald, and R. T. Phren. 1964. Differential Toxicity Response of Normal and Neoplastic Cells *In Vitro* to 3,4-Benzopyrene and 3-Methylcholanthrene. Br. J. Cancer 18:159-164.

Allen, M. J., E. Boyland, C. E. Dukes, E. S. Horning, and J. G. Watson. 1957. Cancer of the Urinary Bladder Induced in Mice with Metabolites of Aromatic Amines and Tryptophan. Br. J. Cancer 11:212-230.

Andervont, H. B. 1950. Induction of Hemangio-endotheliomas and Sarcomas in Mice with *O*-Aminoazotoluene. J. Natl. Cancer Inst. 10:927-941.

Andervont, H. B. 1958. Induction of Hepatomas in Strain C3H Mice with 4-*O*-Tolylazo-*O*-Toluidine and Carbon Tetrachloride. J. Natl. Cancer Inst. 20:431-438.

Andervont, H. B., and T. B. Dunn. 1962. Occurrence of Tumors in Wild House Mice. J. Natl. Cancer Inst. 28:1153-1163.

Andervont, H. B., H. G. Grady, and J. E. Edwards. 1942. Induction of Hepatic Lesions, Hepatomas, Pulmonary Tumors and Hemangio-endotheliomas in Mice with *O*-Aminoazotoluene. J. Natl. Cancer Inst. 3:131-153.

Andervont, H. B., and M. B. Shimkin. 1940. Biologic Testing of Carcinogens. II. Pulmonary-Tumor-Induction Technique. J. Natl. Cancer Inst. 1:225-239.

Andreescheva, N. 1969. Characteristics and Criteria of the Toxic Effects of Certain Nitro and Amino Derivatives of Benzene. Hyg. Sanit. 35(4):51-56.

Andrianova, M. 1971. Transplacental Action of 3-Methylcholanthrene and Benz(α)pyrene on Four Generations of Mice. Bull. Exp. Biol. Med. (USSR) 71(1):677-680.

Argus, M. F., and C. Hoch-Ligeti. 1963. Induction of Malignant Tumors in the Guinea Pig by Oral Administration of Diethylnitrosamine. J. Natl. Cancer Inst. 30:533-551.

Arnold, D., S. Charbonneau, Z. Zawidzka, and H. Grice. 1977. Monitoring Animal Health During Chronic Toxicity Studies. J. Environ. Pathol. Toxicol. 1:227-239.

Axelrad, A., and H. van der Gaag. 1962. Susceptibility to Lymphoma Induction by Gross' Passage A Virus in C3Hf/Bi Mice of Different Ages: Relation to Thymic Cell Multiplication and Differentiation. J. Natl. Cancer Inst. 28:1065-1094.

Baba, T., and S. Takayama. 1961. Influence of p-Dimethylamine-Azobenzene (DAB) Pretreatment in Neonatal Stage on the DAB Heptacarcinogenesis in Adult Rats, with Special Reference to Sex Difference in the Carcinogenesis. Gann 52:73-82.

Barnes, J., and F. Denz. 1954. Experimental Methods in Determining Chronic Toxicity. Pharmacol. Rev. 6:191-242.

Beckers, A., and H. Bazin. 1978. Incidence of Spontaneous Ileocecal Immunocytomas in Hybrids of LOU/C Rats and Rat Strains with Low Spontaneous Tumor Incidence (brief communication). J. Natl. Cancer Inst. 60(6):1505-1508.

Benitz, K. F. 1970. Measurement of Chronic Toxicity. In: Methods in Toxicology, G. E. Paget, ed. F. A. Davis Company, Philadelphia. pp. 82-131.

Berenblum, I., L. Boiato, and N. Trainin. 1966. On the Mechanism of Urethane Leukemogenesis in Newborn C57BL Mice. II. Influence of Thymectomy and of Subsequent Thymus Reimplantation. Cancer Res. 26:361-363.

Bhisey, R. A., and S. M. Sirsat. 1975. Histological Changes in the Mouse Skin After One Subcutaneous Injection of 20-Methylcholanthrene. Indian J. Cancer 12:430-438.

Bielschowsky, F., and W. S. Bullough. 1949. Epidermal Mitotic Activity and the Induction of Skin Tumors in Mice. Br. J. Cancer 3:282-285.

Bonser, G. M., E. Boyland, E. R. Busby, D. B. Clayson, P. L. Grover, and J. W. Jull. 1963. A Further Study of Bladder Implantation in the Mouse as the Means of Detecting Carcinogenic Activity: Use of Crushed Paraffin Wax or Stearic Acid as the Vehicle. Br. J. Cancer 17:127-136.

Bonser, G. M., D. B. Clayson, J. W. Jull, and L. N. Pyrah. 1952. The Carcinogenic Properties of 2-Amino-1-Naphthol Hydrochloride and Its Parent Amine 2-Naphthylamine. Br. J. Cancer 6:412-424.

Boorman, G. A., and C. F. Hollander. 1974. High Incidence of Spontaneous Urinary Bladder and Ureter Tumors in the Brown Norway Rat (brief communication). J. Natl. Cancer Inst. 52(3):1005-1008.

Boyd, E. 1968. Predictive Drug Toxicity: Assessment of Drug Safety Before Human Use. Can. Med. Assoc. J. 98:278-293.

Braun, W., L. Sung, D. Keyes, and R. Kociba. 1977. Pharmacokinetic and Toxicological Evaluation of Dogs Fed 1,2,4,5-Tetrachlorobenzene in the Diet for Two Years. Toxicol. Appl. Pharmacol. 41:218-219.

Bridges, B. A. 1976. Short Term Screening Tests for Carcinogens. Nature 261:195-200.

Brune, H.F.K. 1977. Experimental Results with Percutaneous Applications of Automobile Exhaust Condensates in Mice. In: Air Pollution and Cancer in Man, U. Mohr, D. Schmall, and L. Tomatis, eds. International Agency for Research on Cancer, Lyon, France. pp. 41-47.

Brusick, D. J. 1978. The Role of Short-Term Testing in Carcinogen Detection. Chemosphere No. 5:403-417.

Burek, J. D., and C. F. Hollander. 1977. Incidence Patterns of Spontaneous Tumors in BN/Bi Rats. J. Natl. Cancer Inst. 58(1):99-105.

Butler, W. H. 1979. Report of the Workshop on Carcinogenicity. J. Toxicol. Environ. Health 5:161-162.

Cameron, G. R., and K. K. Cheng. 1951. Failure of Oral DDT to Induce Toxic Changes in Rats. Br. Med. J. 2:819-821.

Campbell, J. S., Y. H. Yang, and J. D. Bolton. 1965. Murine Lung Adenomas Induced by Intravaginal Carcinogen. Arch. Path. 79:588-594.

Cardesa, A., P. Pour, J. Althoff, and U. Mohr. 1974. Effects of Intraperitoneal Injections of Dimethyl- and Diethylnitrosamine, Alone or Simultaneously on Swiss Mice. Z. Krebsforsch. 82:233-238.

Carpanini, F., K. Butterworth, I. Gaunt, I. Kiss, P. Grasso, and S. Gangoli. 1978. Long-Term Toxicity Studies on Chocolate Brown HT in Rats. Toxicology 11:303-307.

Case, M., J. Smith, and R. Nelson. 1978. Acute Mouse and Chronic Dog Toxicity Studies in Danthron Dioctyl Sodium Sulfosuccinate, Poloxalkol and Combinations. Drug. Chem. Toxicol. 1(1):89-101.

Chieco-Bianchi, L., G. De Benedictis, G. Tridente, and L. Fiore-Donati. 1963. Influence of Age on Susceptibility to Liver Carcinogenesis and Skin Initiating Action by Urethane in Swiss Mice. Br. J. Cancer 17:672-680.

Clapp, N. K., R. L. Tyndall, and J. A. Otten. 1971. Differences in Tumor Types and Organ Susceptibility in BALB/c and RF Mice Following Dimethylnitrosamine and Diethylnitrosamine. Cancer Res. 31:196-198.

Clark, D. 1977. Long-Term Inhalation Toxicology Studies. In: Current Approaches in Toxicology, B. Ballantyne, ed. John Wright and Sons, Bristol. pp. 105-114.

Coate, W., W. Schoenfisch, T. Lewis, and W. Busey. 1977. Chronic Inhalation Exposure of Rats, Rabbits, and Monkeys to 1,2,4-Trichlorobenzene. Arch. Environ. Health 32(6):249-255.

Crocker, T. T., J. E. Chase, S. A. Wells, and L. L. Nunes. 1970. Preliminary Report on Experimental Squamous Carcinoma of the Lung in Hamsters and in a Primate. In: Morphology of Experimental Respiratory Carcinogenesis, P. Nettesheim, M. G. Hanna, Jr., and J. W. Deatheridge, Jr., eds. Oak Ridge, Tenn., USAEC Division of Technical Information. pp. 317-328.

Dale, M. M., G. C. Easty, R. Tchao, H. Desai, and M. Andjargholi. 1973. The Induction of Tumors in the Guinea Pig with Methylcholanthrene and Diethylnitrosamine and Their Propogation *In Vivo* and *In Vitro*. Br. J. Cancer 27:445-450.

Davies, R. F., I. R. Major, and E. R. Aberdeen. 1970. Pulmonary Adenomata Induced by Carcinogen Treatment in Organ Culture. Influence of Increasing Amounts of Carcinogen. Br. J. Cancer 24:785-787.

Davies, R. F., I. R. Major, and E. R. Aberdeen. 1971. The Induction of Adenomata in Mouse Lung Homografts by Chemical Carcinogens. Br. J. Cancer 25:565-567.

Davis, B. R., J. K. Whitehead, M. E. Gill, P. N. Lee, A. D. Butterworth, and F.J.R. Roe. 1975. Response of Rat Lung to 3,4-Benzpyrene Administered by Intratracheal Instillation in Infusine With or Without Carbon Black. Br. J. Cancer 31:443-452.

Davis, K., A. Nelson, R. Zwickey, W. Hansen, and O. Fitzhugh. 1966. Chronic Toxicity of Ponceau SX to Rats, Mice, and Dogs. Toxicol. Appl. Pharmacol. 8:306-317.

De Benedictis, G., G. Maiorano, L. Chieco-Bianchi, and L. Fiore-Donati. 1962. Lung Carcinogenesis by Urethane in Newborn, Suckling, and Adult Swiss Mice. Br. J. Cancer 16:686-689.

Deichmann, W. B., M. Keplinger, F. Sala, and E. Glass. 1967. Synergism Among Oral Carcinogens. IV. The Simultaneous Feeding of Four Tumorigens to Rats. Toxicol. Appl. Pharmacol. 11:88-103.

Deichmann, W. B., and J. L. Radomski. 1963. The Carcinogenicity and Metabolism of 2-Naphthylamine and Related Compounds. Ind. Med. Surg. 32:161.

Deichmann, W. B., J. L. Radomski, E. Glass, W.A.D. Anderson, M. M. Coplan, and F. M. Woods. 1965. Synergism Among Oral Carcinogens. III. Simultaneous Feeding of Four Bladder Carcinogens to Dogs. Ind. Med. Surg. 34:640-649.

Della Porta, G. 1968. Use of Newborn and Infant Animals in Carcinogenicity Testing. Food Cosmet. Toxicol. 6:243-252.

Della Porta, G., L. Kolb, and P. Shubik. 1958. Induction of Tracheobronchial Carcinomas in the Syrian Golden Hamster. Cancer Res. 18:592-597.

Della Porta, G., M. Rappaport, U. Saffiotti, and P. Shubik. 1956. Induction of Melanotic Lesions During Skin Carcinogenesis in Hamsters. Arch. Pathol. 61:303-313.

Della Porta, G., and B. Terracini. 1969. Chemical Carcinogenesis in Infant Animals. Prog. Exp. Tumor Res. 11:334-363.

DiPaolo, J. A., R. L. Nelson, P. J. Donovan, and C. H. Evans. 1973. Host Mediated *In Vivo - In Vitro* Assay for Chemical Carcinogenesis. Arch. Pathol. 95:380-385.

Diwan, B. A., and H. Meier. 1974. Strain- and Age-Dependent Transplacental Carcinogenesis by 1-Ethyl-1-Nitrosourea in Inbred Strains of Mice. Cancer Res. 34:764-770.

Doell, R., and W. Carnes. 1962. Urethane Induction of Thymic Lymphoma in C57BL Mice. Nature 194:588-589.

Drozdz, M., E. Kucharz, and M. Kaminski. 1977. Histopathological and Histochemical Studies of the Skin of Guinea Pigs After Long-Term Exposure to Nitrogen Dioxide. Exp. Pathol. Board 14:162-170.

Durham, W. F., P. Ortega, and W. J. Hays, Jr. 1963. The Effect of Various Dietary Levels of DDT on Liver Function, Cell Morphology and DDT Storage in the Rhesus Monkey. Arch. Int. Pharmacodyn. 141:111-129.

Dyer, H., M. Kelly, and R. O'Gara. 1966. Lack of Carcinogenic Activity and Metabolic Fate of Fluorenylacetamide in Monkeys. J. Natl. Cancer Inst. 36:305-322.

Epstein, S., J. Andrea, and H. Jaffe. 1967. Carcinogenicity of the Herbicide Maleic Hydrazide. Nature (London) 215:1388-1390.

Fancher, O. 1978. Species Selection and Animal Models for Toxicologic Studies. Clin. Toxicol. 12(2):239-247.

Farber, E. 1963. Ethionine Carcinogenesis. Adv. Cancer Res. 7:383-474.

Fare, G. 1966. Rat Skin Carcinogenesis by Topical Applications of Some Azo Dyes. Cancer Res. 26:2406-2408.

Federal Register. 1978. Proposed Guidelines for Registering Pesticides in the U.S. Hazard Evaluation: Humans and Domestic Animals. 43FR(163): 37336-37403.

Federal Register. 1979. Proposed Health Effects Tests Standards for Toxic Substances Control Act Test Rules. 44FR(91):27334-27375.

Flaks, A. 1965. The Effect of 9,10-Dimethyl-1,2-Benzanthracene on Young Mice of Low and High Cancer Strain. Br. J. Cancer 19:547-550.

Flaks, A. 1966. Test for Carcinogenesis of Cigarette Tobacco Smoke Condensate Using Young Strong A and C57BL Mice. Br. J. Cancer 20:145-147.

Food Safety Council. 1978. Proposed System for Food Safety Assessment. Food Cosmet. Toxicol. 16, Suppl. 2:1-136.

Fox, J. G. 1977. Clinical Assessment of Laboratory Rodents on Long-Term Bioassay Studies. J. Environ. Path. and Toxicol. 1:199-226.

Frankel, H., R. Yamamoto, E. Weisburger, and J. Weisburger. 1970. Chronic Toxicity of Azathioprine and the Effect of this Immuno-suppressant on Liver Tumor Induction by the Carcinogen *N*-Hydroxy-*N*-2-Fluorenylacetamide. Toxicol. Appl. Pharmacol. 17:462-480.

Friedman, L. 1973. Problems of Evaluating the Health Significance of the Chemicals Present in Foods. In: Pharmacology and the Future of Man, Proc. 5th Int. Congr. Pharmacology, San Francisco, 1972, Vol. 2. Karger, New York. pp. 30-41.

Fugmann, R. A., J. C. Anderson, R. L. Stolfi, and D. S. Martin. 1977. Comparison of Adjuvant Chemotherapeutic Activity Against Primary and Metastatic Spontaneous Murine Tumors. Cancer Res. 37:496-500.

Futami, T., Y. Samata, T. Tsuda, and F. Masuda. 1977. Toxicity Study on Sodium Polyalkylarylsulfonate (Na-PAAS) in Rats: II. Chronic Toxicity Study. Oyo Yakuri 13(1):147-159.

Gage, J. 1970. Experimental Inhalation Toxicity. In: Methods in Toxicology, G. Paget, ed. F. A. Davis Company, Philadelphia. pp. 258-277.

Gak, J. C., C. Graillot, and R. Truhaut. 1976. Use of the Golden Hamster in Toxicology (Part II of two parts). Lab. Anim. Sci. 26(2):274-280.

Gargus, J., O. Paynter, and W. Reese, Jr. 1969. Utilization of Newborn Mice in the Bioassay of Chemical Carcinogens. Toxicol. Appl. Pharmacol. 15:552-559.

Gilbert, C., J. Gillman, P. Loustalot, and W. Lutz. 1958. The Modifying Influence of Diet and the Physical Environment on Spontaneous Tumor Frequency in Rats. Br. J. Cancer 12:565-593.

Giurgea, M. 1977. Peracetam: Toxicity and Reproduction Studies. Farmaco Ed. Prat. 32:48-52.

Glucksmann, A., and C. Cherry. 1971. Sex Difference and Carcinogenic Dosage in the Induction of Neoplasms in the Salivary Glands of Rats. Br. J. Cancer 25:212-224.

Goerttler, K., and H. Lohrke. 1977. Diaplacental Carcinogenesis: Tumor Localization and Tumor Incidence in NMRI Mice After Diaplacental Initiation with DMBA and Urethane and Postnatal Promotion with the Phorbol Ester TPA in a Modified 2-Stage Berenblum/Mottram Experiment. Virchos Arch. A. Path. Anat. and Histol. 376:117-132.

Golberg, L., ed. 1974. Carcinogenesis Testing of Chemicals. The Chemical Rubber Company, Cleveland, Ohio. 144 pp.

Golberg, L. 1975. Safety Evaluation Concepts. J. Assoc. Off. Anal. Chem. 58(4):635-644.

Goldenthal, E. 1968. Current Views on Safety Evaluation of Drugs. U.S. Food and Drug Administration Papers May:13-18.

Goldwater, L. J., A. J. Rosso, and M. Kleinfeld. 1965. Bladder Tumors in a Coal Tar Dye Plant. Arch. Environ. Health 11:814-817.

Gorrod, J., R. Carter, and F. Roe. 1968. Induction of Hepatomas by 4-Aminobiphenyl and Three of Its Hydroxylated Derivatives Administered to Newborn Mice. J. Natl. Cancer Inst. 41:403-410.

Graffi, A., F. Hoffman, and M. Schutt. 1967. *N*-Methyl-*N*-Nitrosourea As a Strong Topical Carcinogen When Painted on the Skin of Rodents. Nature (London) 214:611.

Grahn, D., and K. F. Hamilton. 1964. Influence of Sex, Environment, and Radiation Factors on Life Shortening and Tumor Incidence in C3H Mice. Radiat. Res. 22:191.

Grasso, P. 1976. Review of Tests for Carcinogenicity and Their Significance to Man. Clin. Toxicol. 9:745-760.

Gray, J., R. Weaver, J. Bollert, and E. Feenstra. 1972. The Oral Toxicity of Clindamycin in Laboratory Animals. Technol. Appl. Pharmacol. 21:516-531.

Greenman, D. L., and R. R. Delongchamp. 1979. Variability of Response to Diethylstilbesterol: A Comparison of Inbred with Hybrid Mice. J. Toxicol. and Environ. Health 5:131-143.

Greenman, D., R. Delongchamp, and B. Highman. 1979. Variability of Response to Diethylstilbestrol: A Comparison of Inbred with Hybrid Mice. J. Toxicol. Environ. Health 5:131-143.

Greenwald, P., J. Barlow, P. Nasca, and W. Burnett. 1971. Vaginal Cancer After Maternal Treatment with Synthetic Estrogens. New Engl. J. Med. 285:390-392.

Griesemer, R. A., P. Nettesheim, D. H. Martin, and J. E. Caton, Jr. 1977. Quantitative Exposure of Grafted Rat Tracheas to 7,12-Dimethylbenz(*a*)-anthracene. Cancer Res. 37:1266-1271.

Griswold, D. P., A. E. Casey, E. K. Weisburger, and J. H. Weisburger. 1968. The Carcinogenicity of Multiple Intragastric Doses of Aromatic and Heterocyclic Nitro or Amino Derivatives in Young Sprague-Dawley Rats. Cancer Res. 28:924-933.

Gruenstein, M., D. R. Meranze, and M. B. Shimkin. 1966. Mammary, Sebaceous, and Cutaneous Neoplasms and Leukemia in Male Wistar Rats Receiving Repeated Gastric Instillations of 3-Methylcholanthrene. Cancer Res. 26:2202-2205.

Haag, H., J. Finnegan, P. Larson, M. Dreyfuss, R. Main, and W. Riese. 1948. Comparative Chronic Toxicity for Warm-Blooded Animals of 2,3-bis-(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT). Ind. Med. 17:477-484.

Haun, C. 1975. Toxicity of High Density Jet Fuel Components. AMRL-TR-75-125, Aerospace Medical Division, Wright-Patterson Air Force Base, Ohio.

Hayes, W. 1967. Toxicity of Pesticides to Man: Risks from Present Levels. Proc. R. Soc. London Ser. B. 167:101-127.

Herbst, A., L. Ulfelder, and D. Poscanzer. 1971. Adenocarcinoma of the Vagina. Association of Maternal Stilbestrol Therapy with Tumor Appearance in Young Women. New Engl. J. Med. 284:878-881.

Herrold, K. M. 1964*a*. Induction of Olfactory Neuroepithelial Tumors in Syrian Hamsters by Diethylnitrosamine. Cancer 17:114-121.

Herrold, K. 1964*b*. Effect of Route of Administration on the Carcinogenic Action of Diethylnitrosamine. Br. J. Cancer 18:763-767.

Herrold, K. 1969. Aflatoxin Induced Lesions in Syrian Hamsters. Br. J. Cancer 23:655-660.

Herrold, K. M., and L. J. Dunham. 1963. Induction of Tumors in the Syrian Hamster with Diethylnitrosamine (*N*-Nitrosodiethylamine). Cancer Res. 23:773-777.

Heston, W. E. 1958. Mammary Tumors in Agent-Free Mice. Ann. N.Y. Acad. Sci. 71:931-942.

Heston, W. E., and G. Vlahakis. 1966. Factors in the Causation of Spontaneous Heptaomas in Mice. J. Natl. Cancer Inst. 37:839-843.

Heston, W. E., and G. Vlahakis. 1968. C3H-A^{vy} - A High Hepatoma and High Mammary Tumor Strain of Mice. J. Natl. Cancer Inst. 40:1161-1166.

Heston, W. E., G. Vlahakis, and M. K. Deringer. 1960. High Incidence of Spontaneous Hepatomas and the Increase of This Incidence with Urethane in C3H, C3Hf, and C3He Male Mice. J. Natl. Cancer Inst. 24:425-435.

Heuper, W. C., and H. D. Wolf. 1937. Experimental Production of Aniline Tumors of Dogs in the Bladder. Amer. J. Pathol. 13:656-657.

Hiaro, F., T. Fujisawa, E. Tsubura, K. Akamatsu, Y. Yamamura. 1968. Experimental Lung Cancer in Rabbits Induced by Chemical Carcinogens. Gann 59:497-505.

Hoag, W. G. 1963. Spontaneous Cancer in Mice. Ann. N.Y. Acad. Sci. 108:805-831.

Hodge, H., A. Boyce, W. Deichmann, and H. Kraybill. 1967. Toxicology and No-Effect Levels of Aldrin and Dieldrin. Toxicol. Appl. Pharmacol. 10:613-675.

Holland, J. M., D. G. Gosslee, and N. J. Williams. 1979. Epidermal Carcinogenicity of Bis(2,3-epoxycyclopentyl)ether, 2,2-Bis(β-glycidyloxyphenyl) propane and m-Phenylenediamine in Male and Female C3H and C57 BL/6 Mice. Cancer Res. 39:1714-1721.

Homburger, F., and J. R. Baker. 1969. Accelerated Carcinogen Testing. Progr. Exp. Tumor Res., Vol. 11. Karger, New York. pp. 384-394.

Homburger, F., and S. S. Hsuch. 1970. Rapid Induction of Subcutaneous Fibrosarcoma by 7,12-Dimethylbenz(a)anthracene in an Inbred Line of Syrian Hamsters. Cancer Res. 30:1449-1452.

Homburger, F., S. Hsuch, C. S. Kerr, and A. B. Russfield. 1972. Inherited Susceptibility of Inbred Strains of Syrian Hamsters to Induction of Subcutaneous Sarcomas and Mammary and Gastrointestinal Carcinomas by Subcutaneous and Gastric Administration of Polynuclear Hydrocarbons. Cancer Res. 32:360-366.

Homburger, F., A. B. Russfield. 1970. An Inbred Line of Syrian Hamsters with Frequent Spontaneous Adrenal Tumors. Cancer Res. 30:305-308.

Homburger, F., and A. Tregier. 1960. Modifying Factors in Carcinogenesis. Prog. Exptl. Tumor Res. 1:311.

Howell, J. 1963. The Experimental Production of Vascular Tumors in the Rat. Br. J. Cancer 17:663-671.

Huggins, C., L. C. Grand, and F. P. Brillantes. 1961. Mammary Cancer Induced by a Single Feeding of Polynuclear Hydrocarbons and Its Suppression. Nature 189:204-207.

Huggins, C., L. Grand, and R. Fukunishi. 1964. Aromatic Influences on the Yields of Mammary Cancers Following Administration of 7,12-Dimethylbenz(*a*)anthracene. Proc. Natl. Acad. Sci., Vol. 51. pp. 737-742.

Ihle, J. N., L. O. Arthur, and D. L. Fine. 1976. Autogenous Immunity to Mouse Mammary Tumor Virus in Mouse Strains of High and Low Mammary Tumor Incidence. Cancer Res. 36:2840-2844.

Innes, J.R.M., B. M. Ulland, M. G. Valerio, L. Petrucelli, L. Fishbein, E. R. Hart, A. J. Pallotta, R. R. Bates, H. L. Falk, T. J. Gart, M. Klein, I. Mitchell, and J. Peters. 1969. Bioassay of Pesticides and Industrial Chemicals for Tumorigenicity in Mice. J. Natl. Cancer Inst. 42:1101-1114.

Ivankovic, S. 1973. Experimental Prenatal Carcinogenesis. In: Transplacental Carcinogenesis, L. Tomatis and U. Mohr, eds. Scientific Publication No. 4, International Agency for Research on Cancer, Lyon, France. pp. 92-99.

Jalanko, H., I. Virtanen, E. Engvall, and E. Ruoslahti. 1978. Early Increase of Serum Alpha-Fetoprotein in Spontaneous Hepatocarcinogenesis in Mice. Int. J. Cancer 21(4):453-459.

Jull, J. W. 1951. The Induction of Tumors of the Bladder Epithelium in Mice by the Direct Application of a Carcinogen. Br. J. Cancer 5:328-330.

Jungherr, E. 1963. Spontaneous Cancer in Zoo and Laboratory Animals. Part II. Tumors and Tumor-Like Conditions in Monkeys. Ann. N.Y. Acad. Sci. 108:777-792.

Kadota, T., H. Kohda, and J. Miyamoto. 1975. Subchronic Toxicity Studies of Sumithion, Sumioxon, and *p*-Nitrocresol in Rats and 92-Week Feeding Study of Sumithion with Special Reference to Change of Cholinesterase Activity. Botyu-Kagaku 40:38-48.

Kaiser, J. 1964. A One-Year Study of the Toxicity of Ethambutol in Dogs: Results During Life. Toxicol. Appl. Pharmacol. 6:577-567.

Kaplan, A., and H. Sherman. 1977. Toxicity Studies with Methyl-*N*-[(Methyllamine-carbonyl)oxyl]-ethanimidothioate. Toxicol. Appl. Pharmacol. 40:1-17.

Kaye, A., and N. Trainin. 1966. Urethan Carcinogenesis and Nucleic Acid Metabolism: Factors Influencing Lung Adenoma Induction. Cancer Res. 26:2206-2212.

Kello, D., and K. Kostial. 1973. The Effect of Milk Diets on Lead Metabolism in Rats. Environ. Res. 6:355-360.

Kelly, M., and R. O'Gara. 1961. Induction of Tumors in Newborn Mice with Dibenz(*a*)anthracene and 3-Methylcholanthrene. J. Natl. Cancer Inst. 26:651-679.

Kelly, M. G., R. W. O'Gara, R. H. Adamson, K. Gadekar, C. C. Botkin, W. H. Reese, Jr., and W. T. Kerber. 1966. Induction of Hepatic Cell Carcinomas in Monkeys with *N*-Nitrosodiethylamine. J. Natl. Cancer Inst. 36:323-351.

Ketkar, M., G. Reznik, P. Schneider, and U. Mohr. 1978. Investigations on the Carcinogenic Burden by Air Pollution in Man. Intratracheal Instillation Studies with Benzo(*a*)pyrene in Bovine Serum Albumin in Syrian Hamsters. Cancer Lett. 4:235-239.

Kitagawa, K., M. Wakakura, and S. Ishikawa. 1977. Light Microscopic Study of Endocrine Organs of Rats Treated by Carbamate Pesticide. J. Toxicol. Sci. 2:53-60.

Klein, M. 1951. The Action of Croton Oil in the Induction of Sarcomas in Mice. J. Natl. Cancer Inst. 11:843-848.

Klein, M. 1952. The Action of Methylcholanthrene, Croton Oil, and 1,2-Benzanthracene in the Induction of Skin Tumors in Strain DBA Mice. J. Natl. Cancer Inst. 12:735-742.

Klein, M. 1953. Effects of Croton Oil on Induction of Tumors by 1,2-Benzanthracene, Deoxycholic Acid, or Low Doses of 20-MCA in Mice. J. Natl. Cancer Inst. 13:333.

Klein, M. 1963. Susceptibility of Strain BLoAF$_1$/J Hybrid Infant Mice to Tumorigenesis with 1,2-Benzanthracene, Deoxycholic Acid, and 3-Methylcholanthrene. Cancer Res. 23(6):1701-1707.

Kociba, R., S. McCollister, C. Park, T. Torkelson, and P. Gehring. 1974. 1,4-Dioxane Toxicity as Determined by a Two-Year Dose-Response Study in Rats. Toxicol. Appl. Pharmacol. 29:36.

Kociba, R., B. Schwetz, D. Keyes, G. Jersey, J. Ballard, D. Dittenber, J. Quast, C. Wade, and C. Humiston. 1977. Chronic Toxicity and Reproduction Studies of Hexachlorobutodiene in Rats. Environ. Health Perspect. 21:49-53.

Koelle, G., and A. Gilman. 1946. The Chronic Toxicity of Diisopropyl Fluorophosphate (DPF) in Dogs, Monkeys, and Rats. J. Pharmacol. Exp. Ther. 87:435-448.

Koss, G., S. Seubert, A. Seubert, W. Koransky, and H. Ippen. 1978. Studies on the Toxicology of Hexachlorobenzene. III. Observations in a Long-Term Experiment. Arch. Toxicol. 40:285-294.

Krasovskii, G. 1976. Extrapolation of Experimental Data from Animals to Man. Environ. Health Perspect. 13:51-58.

Kroes, R., G. M. Williams, and J. H. Weisburger. 1973. Early Appearance of Serum α-Fetoprotein as a Function of Dosage of Various Hepatocarcinogens. Cancer Res. 33:613-617.

Kuschner, M., S. Laskin, E. Cristofano, and N. Nelson. 1957. Experimental Carcinoma of the Lung. In: Proceedings of the Third National Cancer Conference. Lippincott, Philadelphia. pp. 485-495.

Laishes, B., and E. Farber. 1978. Transfer of Viable Putative Preneoplastic Hepatocytes to the Livers of Syngenic Host Rats. J. Natl. Cancer Inst. 61(2):507-512.

Laishes, B. A., L. Fink, and B. I. Carr. 1980. A Liver Colony Assay for a New Hepatocyte Phenotype as a Step Towards Purifying New Cellular Phenotypes That Arise During Hepatocarcinogenesis. Ann. N.Y. Acad. Sci. (in press).

Lawrence, W. 1976. Systemic Toxicity. Acute vs. Subchronic and Chronic. Drug. Cosmet. Ind. November 1976:40-114.

Laws, J. O., and A. Flaks. 1966. Pulmonary Adenomata Induced by Carcinogen Treatment in Organ Culture. Br. J. Cancer 20:550-554.

Lee, C., J. Bhandari, J. Winston, W. House., P. Peters, R. Dixon, and J. Woods. 1977. Inhalation Toxicity of Vinyl Chloride and Vinylidene Chloride. Environ. Health Perspect. 21:25-32.

Lee, C-C., J. Q. Russell, and J. L. Minor. 1978. Oral Toxicity of Ferric Dimethyl-Dithiocarbamate (Ferbam) and Tetramethylthiuram Disulfide (Thiram) in Rodents. J. Toxicol. Environ. Health 4:93-106.

Lee, K., B. Toth, and P. Shubik. 1963. Carcinogenic Response of the Syrian Golden Hamster Treated at Birth with 7,12-Dimethylbenz(*a*)-anthracene. Proc. Soc. Exptl. Biol. Med. 114:579-582.

Levinsky, H., H. MacFarland, B. Procter, G. Rona, and A. Blair. 1978. A Study of the Chronic Toxicity of Inhaled Disodium Cromoglycate in the Squirrel Monkey. Toxicol. Appl. Pharmacol. 45:141-153.

Levy, B M. 1963. Induction of Fibrosarcoma in the Primate, *Tamarinus nigricollis*. Nature 200:182-183.

Liebelt, R., A. Liebelt, and M. Lane. 1964. Hormonal Influences on Urethan Carcinogenesis in C3H/f Mice. Cancer Res. 24:1869-1879.

Liebelt, R., R. Yoshida, and G. Gray. 1961. Enhancement of Liver Tumorigenesis in Zb Mice Injected with Urethan at Newborn Age. Proc. Assoc. Cancer Res. 3:245.

Lijinsky, W., A. Ferrero, R. Montesano, and C.E.M. Wenyon. 1970. Tumorigenicity of Cyclic Nitrosamines in Syrian Golden Hamsters. Z. Krebsforsch. 74:185-189.

Lijinsky, W., K. Lee, Y. Tomatis, and W. H. Butler. 1967. Nitrosoazetidine — a Potent Carcinogen of Low Toxicity. Naturwissenschaften 54:518.

Lijinsky, W., and H. W. Taylor. 1978. Relative Carcinogenic Effectiveness of Derivatives of Nitrosodiethylamine in Rats. Cancer Res. 38: 2391-2394.

Littlefield, N., and R. Kodell. 1979. Influence of Genetic Population Structure on the Results of Chronic Toxicity Studies. J. Toxicol. Environ. Health 5:121-129.

Loomis, T. 1974. Essentials of Toxicology, 2nd ed., Lea and Febiger, Philadelphia. pp. 189-191.

Luz, A. 1977. The Range of Incidence of Spontaneous Neoplastic and Non-Neoplastic Lesions of the Laboratory Mouse. Z. Versuchstierk. 19(6): 342-343.

Maekawa, A., and S. Odashima. 1975. Spontaneous Tumors in ACl/N Rats. J. Natl. Cancer Inst. 55:1437-1445.

Magee, P. 1970. Tests for Carcinogenic Potential. In: Methods in Toxicology, G. Paget, ed. F. A. Davis Company, Philadelphia. pp. 158-196.

McCollister, S., R. Kociba, C. Humiston, D. McCollister, and P. Gehring. 1974. Studies of the Acute and Long-Term Oral Toxicity of Chlorpyrifos (*o*,*o*-Diethyl-*o*-(3,5,6-Trichloro-2-Pyridyl)Phosphorothioate). Food Cosmet. Toxicol. 12:45-61.

McNamara, B. 1976. Concepts in Health Evaluation of Commercial and Industrial Chemicals. In: Advances in Modern Toxicology, Part 1, Vol. 1, M. Mehlman, R. Shapiro, and H. Blumenthal, eds. John Wiley and Sons, New York. pp. 61-140.

Milievskaja, I. L., and N. S. Kiseleva. 1976. Comparative Study of the Carcinogenic Activities of NAS and Some Chemical Carcinogens When Introduced into the Buccal Pouch of the Syrian Hamster. Bull. World Health Organ. 54:607-614.

Miller, E. C., J. A. Miller, and M. Enomota. 1964. The Comparative Carcinogenicities of 2-Acetylaminofluorene and Its *N*-Hydroxy Metabolite in Mice, Hamsters and Guinea Pigs. Cancer Res. 24:2018-2031.

Ministry of Health and Welfare Canada. 1975. The Testing of Chemicals for Carcinogenicity, Mutagenicity, and Teratogenicity. Ministry of Health and Welfare, Ottawa. 183 pp.

Misra, A., and S. Mule. 1977. Severe Toxicity and Lethality in Some Monkeys Following Chronic Administration of *l*-α-Acetylmethadol (LAAM). Am. J. Drug. Alcohol Abuse 4(3):431-440.

Miyoshi, K., and S. Takauchi. 1977. Chronic Tellurium Intoxication in Rats. Folia Psychiatr. Neurol. Jpn. 31(1):111-118.

Mohr, U. 1973. Effects of Diethylnitrosamine on Fetal and Suckling Syrian Golden Hamsters. In: Transplacental Carcinogenesis, L. Tomatis and U. Mohr, eds. Scientific Publication No. 4, International Agency for Research on Cancer, Lyon, France. pp. 65-70.

Mohr, U., G. Reznik, and P. Pour. 1977. Carcinogenic Effects of Diisopropanolnitrosamine in Sprague-Dawley Rats. J. Natl. Cancer Inst. 58:361-366.

Molello, J., C. Gerbig, and V. Robinson. 1973. Toxicity of [4,4'-(Isopropylidenedithio)bis(2,6-di-*t*-butylphenol)], Probucol, in Mice, Rats, Dogs, and Monkeys: Demonstration of a Species-Specific Phenomenon. Toxicol. Appl. Pharmacol. 24:590-593.

Morris, H. P., and W. H. Eyestone. 1953. Tumors of the Liver and Urinary Bladder of Dogs After Ingestion of 2-Acetylaminofluorene. J. Natl. Cancer Inst. 13:1139-1165.

Morris, H. P., and H. I. Firminger. 1956. Influence of Sex and Sex Hormones on Development of Hepatomas and Other Hepatic Lesions in Strain AXC Rats Ingesting 2-Diacetylaminofluorene. J. Natl. Cancer Inst. 16:927-949.

Mulay, A. S., and R. W. O'Gara. 1959. Incidence of Liver Tumors in Male and Female Rats Fed Carcinogenic Azo Dyes. Proc. Soc. Exptl. Biol. Med. 100:320-322.

Munro, I. 1977. Considerations in Chronic Toxicity Testing: The Chemical, the Dose, the Design. J. Environ. Pathol. Toxicol. 1:183-197.

Nagasawa, H., R. Yanai, and I. Azuma. 1978. Suppression by *Nocardia rubra* Cell Wall Skeleton of Mammary DNA Synthesis, Plasma Prolactin Level, and Spontaneous Tumorigenesis in Mice. Cancer Res. 38:2160-2162.

National Academy of Sciences. 1975. Principles for Evaluating Chemicals in the Environment. National Academy of Sciences, Washington, D.C. pp. 115-153.

National Academy of Sciences. 1977. Principles and Procedures for Evaluating the Toxicity of Household Substances. National Academy of Sciences, Washington, D.C. 130 pp.

National Institutes of Health. 1968-1973. Survey of Compounds Which Have Been Tested for Carcinogen Activity. Public Health Service Publication No. 149, Government Printing Office, Washington, D.C.

Neiman, J. M. 1968. The Sensitizing Carcinogenic Effect of Small Doses of Carcinogen. Europ. J. Cancer 4:537-545.

Nelson, A. A., O. G. Fitzhugh, and H. O. Calvery. 1943. Liver Tumors Following Cirrhosis Caused by Selenium in Rats. Cancer Res. 3:230-236.

Nelson, A. A., and G. Woodard. 1953. Tumors of the Urinary Bladder, Gall Bladder and Liver in Dogs Fed *O*-Aminoazotoluene or *p*-Dimethylaminoazobenzene. J. Natl. Cancer Inst. 13:1497-1509.

Nettesheim, P., and R. A. Griesemer. 1978. Experimental Models for Studies of Respiratory Tract Carcinogenesis. In: Pathogenesis and Therapy of Lung Cancer, Lung Biology in Health and Disease 10, C. Harris, ed. New York. pp. 75-188.

Nettesheim, P., and A. S. Hammons. 1971. Induction of Squamous Cell Carcinoma in the Respiratory Tract of Mice. J. Natl. Cancer Inst. 47:697-701.

Newberne, P. M., and A. Rogers. 1972. Vitamin A, Liver, Colon Carcinoma in Rats Fed Low Levels of Aflatoxin. Toxicol. Appl. Pharmacol. 22:280.

Nishizuka, Y., K. Nakakuki, and T. Sakakura. 1964. Induction of Pulmonary Tumors and Leukemia by a Single Injection of 4-Nitroquinoline 1-Oxide to Newborn and Infant Mice. Gann 55:495-508.

Noble, R., B. C. Hochachka, and D. King. 1975. Spontaneous and Estrogen-Produced Tumors in Nb Rats and Their Behavior After Transplantation. Cancer Res. 35:766-780.

Norris, J., R. Kociba, B. Schwetz, J. Rose, C. Humiston, L. Jewett, P. Gehring, and J. Mailhes. 1975. Toxicology of Octabromobiphenyl and Decabromodiphenyl Oxide. Environ. Health Perspect. 11:153-161.

Noyes, W. F. 1968. Carcinogen Induced Sarcoma in the Primitive Primate, *Tupaia glis*. Proc. Soc. Exp. Biol. Med. 127:594-596.

Noyes, W. F. 1969. Carcinogen-Induced Neoplasia with Metastasis in a South American Primate, *Saquinus oedipus*. Proc. Soc. Expt. Biol. Med. 131:223-225.

O'Gara, R. W., and M. G. Kelly. 1965. Pulmonary Arteriolitis in Infant Monkeys Produced by Subcutaneous Injections of Polycyclic Hydrocarbons. Arch. Path. (Chicago) 79:475-483.

Oppenheimer, B. S., E. T. Oppenheimer, and A. P. Stout. 1948. Sarcomas Induced by Rats by Implanting Cellophane. Proc. Soc. Expt. Biol. Med. 67:33-34.

Outzen, H. C., R. Custer, R. Phillips, G. J. Eaton, and R. T. Prehn. 1975. Spontaneous and Induced Tumor Incidence in Germ Free Nude Rats. J. Reticuloendothel. Soc. 17(1):1-9.

Page, N. P. 1977*a*. Concepts of a Bioassay Program in Environmental Carcinogenesis. In: Advances in Modern Toxicology, Vol. 3: Environmental Cancer, H. Kraybill and M. Mehlman, eds. Hemisphere Publishing Corp., Washington, D.C. pp. 87-171.

Page, N. 1977*b*. Chronic Toxicity and Carcinogenicity Guidelines. J. Environ. Pathol. Toxicol. 1:161-182.

Peacock, P. M. 1962. A Short-Term Test for Carcinogenicity (The Effects of Certain Closely-Related Polycyclic Aromatic Hydrocarbons on Embryo Tissue Homografts in BALB/c Strain Mice). Br. J. Cancer 16:701-706.

Peacock, P. M., and E. Dick. 1963. A Short-Term Test for Carcinogenicity (Mouse Embryo Tissue Homografts in BALB/c Strain Mice). Br. J. Cancer 17:59-61.

Peck, H. 1968. An Appraisal of Drug Safety Evaluation in Animals and the Extrapolation of Results to Man. In: Importance of Fundamental Principles in Drug Evaluation, D. Tedeschi and R. Tedeschi, eds. Raven Press, New York. pp. 449-471.

Peck, H. 1974. Design of Experiments to Detect Carcinogenic Effects of Drugs. In: Carcinogenesis Testing of Chemicals, L. Goldberg, ed. The Chemical Rubber Company, Cleveland, Ohio. pp. 1-14.

Peirson, M., P. Cheeke, and E. Dickinson. 1977. Resistance of the Rabbit to Dietary Pyrrolizidine (Senecio) Alkaloid. Res. Commun. Chem. Pathol. Pharmacol. 16(3):561-564.

Peraino, C., R.J.M. Fry, and E. Staffeldt. 1973. Enhancement of Spontaneous Hepatic Tumorigenesis in C3H Mice by Dietary Phenobarbital (brief communication). J. Natl. Cancer Inst. 51:1349-1350.

Percy, D. H., and A. M. Jonas. 1971. Incidence of Spontaneous Tumors in CD^{R}-1 H$\bar{a}$M/ICR Mice. J. Natl. Cancer Inst. 46:1045-1053.

Petzold, G. L. and J. A. Swenberg. 1978. Detection of DNA Damage Induced *In Vivo* Following Exposure of Rats to Carcinogens. Cancer Res. 38: 1589-1594.

Pietra, G., H. Rappaport, and P. Shubik. 1961. The Effects of Carcinogenic Chemicals in Newborn Mice. Cancer 14:308-317.

Pietra, G., K. Spencer, and P. Shubik. 1959. Response of Newly Born Mice to a Chemical Carcinogen. Nature 183:1689.

Pollard, M., and M. Kajima. 1970. Lesions in Aged Germ Free Wistar Rats. Amer. J. Pathol. 61:25-32.

Port, C. 1976. Animal Model: Lead Nephropathy in Gerbils Following Short- and Long-Term Administration of Lead Acetate. Am. J. Pathol. 85:519-522.

Pott, F., A. Brockhaus, and F. Huth. 1973. Tests on the Production of Tumors in Animal Experiments with Polycyclic Aromatic Hydrocarbons. Zbl. Bakt. Hyg., I. Abt. Orig. B 157:34-43.

Pott, F., R. Tomingas, and J. Misfeld. 1977. Tumors in Mice After Subcutaneous Injection of Automobile Exhaust Condensates. In: Air Pollution and Cancer in Man, Mohr, Sahmahl, and Tomatis, eds. International Agency for Research on Cancer. pp. 79-87.

Pour, P., F. W. Kruger, and J. Althoff. 1974. Cancer of the Pancreas Induced in the Syrian Golden Hamster. Am. J. Pathol. 76:349-358.

Pour, P., F. W. Kruger, and J. Althoff. 1975. Effect of Beta-oxidized Nitrosamines on Syrian Hamsters. III. 2,2-Dihydroxy-di-*n*-propylnitrosamine. J. Natl. Cancer Inst. 54:141-145.

Pour, P., N. Kmoch, E. Greiser, U. Mohr, J. Althoff, and A. Cardesa. 1976. Spontaneous Tumors and Common Diseases in Two Colonies of Syrian Hamsters. J. Natl. Cancer Inst. 56(6):931-935.

Purchase, I., and J. van der Watt. 1970. The Acute and Chronic Toxicity of Sterigmatocystin. In: Proceedings of the Symposium on Mycotoxins in Human Health, Pretoria, September 2-4, 1970, I. Purchase, ed. MacMillan, New York. pp. 209-213.

Rall, D. 1969. Difficulties in Extrapolating the Results of Toxicity Studies in Laboratory Animals to Man. Environ. Res. 2:360-367.

Reeves, A. L., H. E. Puro, and R. G. Smith. 1974. Inhalation Carcinogenesis from Various Forms of Asbestos. Environ. Res. 8:178-202.

Reuber, M. D. 1976. Effect of Age and Sex on Lesions of the Esophagus in Buffalo Strain Rats Ingesting Diethylnitrosamine. Exp. Cell Biol. 44:65-72.

Reuber, M. D., and E. L. Glover. 1967. Hyperplastic and Early Neoplastic Lesions of the Liver in Buffalo Strain Rats of Various Ages Given Subcutaneous Carbon Tetrachloride. J. Natl. Cancer Inst. 38:891-899.

Reznik, G. 1975. The Carcinogenic Effect of (DMN) on the Chinese Hamster (*Cricetulus Griseus*). Cancer Lett. 1:25-28.

Rice, J. 1976. Carcinogenesis: A Late Effect of Irreversible Toxic Damage During Development. Environ. Health Perspect. 18:133-139.

Rigdon, R. H., M. C. Benge, H. Kirchoff, J. Mack, and J. Neal. 1969. Leukemia in Mice Fed Benzo(*a*)pyrene: A Clinical, Pathologic and Hematologic Study. Tex. Rep. Biol. Med. 27:803-820.

Rigdon, R. H., and J. Neal. 1966. Gastric Carcinomas and Pulmonary Adenomas in Mice Fed Benzo(*a*)pyrene. Tex. Rep. Biol. Med. 24:195-207.

Riley, V. 1975. Mouse Mammary Tumors: Alteration of Incidence as Apparent Function of Stress. Science 189:465-467.

Roe, F. 1975. Neonatal Induction of Hepatic and Other Tumors. In: Mouse Hepatic Neoplasia, W. Butler and P. M. Newbern, eds. Elsevier Scientific Publishing Co., New York. Chapter 7.

Roe, F., R. Carter, and S. Adamthwaite. 1969. Induction of Liver and Lung Tumors in Mice by 6-Aminochrysene Administered During the First Three Days of Life. Nature (London) 221:1063-1064.

Roe, F., B. Mitchley, and M. Walters. 1963. Tests for Carcinogenesis Using Newborn Mice: 1,2-Benzanthracene, 2-Naphthylamine, 2-Naphthylhydroxylamine and Ethyl Methane Sulphonate. Br. J. Cancer 17:255-260.

Roe, F., K. Rowsen, and M. Salaman. 1961. Tumours of Many Sites Induced by Injection of Chemical Carcinogenesis into Newborn Mice, A Sensitive Test for Carcinogenesis: The Implication for Certain Immunological Theories. Br. J. Cancer 15:515-530.

Rosenkrantz, H., R. Sprague, R. Fleischman, and M. Braude. 1975. Oral Δ^9-Tetrahydrocannabinol Toxicity in Rats Treated for Periods up to 6 Months. Toxicol. Appl. Pharmacol. 32:399-417.

Rygaard, J., and C. O. Polvson. 1974. The Mouse Mutant Nude Does Not Develop Spontaneous Tumors. Acta. Pathol. Microbiol. Scand. 82: 99-106.

Sabine, J. R., B. J. Horton, and M. B. Wicks. 1973. Spontaneous Tumors in C3H-A^{vy} and C3H-A^{vy}fB Mice: High Incidence in the United States and Low Incidence in Australia. J. Natl. Cancer Inst. 50:1237-1242.

Saffiotti, U., F. Cefis, R. Montesano, and A. R. Sellakumar. 1966. Induction of Bladder Cancer in Hamsters Fed Aromatic Amines. Ind. Med. Surg. 35:564.

Sass, B., L. S. Rabstein, R. Madison, R. M. Nims, R. L. Peters, and G. J. Kelloff. 1975. Incidence of Spontaneous Neoplasms in F344 Rats Throughout the Natural Life-Span. J. Natl. Cancer Inst. 54:1449-1456.

Schepers, G.W.H. 1971. Lung Tumors of Primates and Rodents. Part II. Inhalation Experiments. Ind. Med. May:23-31.

Schmähl, D., and H. Osswald. 1967. Carcinogenesis in Different Animal Species by Diethylnitrosamine. Experentia 23:497-498.

Schrauzer, G. N., D. A. White, J. E. McGinness, and C. J. Schneider. 1978. Arsenic and Cancer: Effects of Joint Administration of Arsenite and Selenite on the Genesis of Mammary Adenocarcinoma in Inbred Female C_3H/St Mice. Bioinorg. Chem. 9:245-253.

Schreiber, H., K. Schreiber, and D. H. Martin. 1975. Experimental Tumor Induction in a Circumscribed Area of the Hamster Trachea: Correlation of Histology and Exfoliative Cytology. J. Natl. Cancer Inst. 54:187-197.

Sherman, H., and A. Kaplan. 1975. Toxicity Studies with 5-Bromo-3-*sec*-Butyl-6-Methyluracil. Toxicol. Appl. Pharmacol. 34:189-196.

Shimkin, M. B. 1940. Induced Pulmonary Tumors in Mice. II. Reaction of Lungs of Strain A Mice to Carcinogenic Hydrocarbons. Arch. Path. 29:239-255.

Shimkin, M. B., and G. D. Stoner. 1975. Lung Tumors in Mice: Application to Carcinogenesis Bioassay. Adv. Cancer Res. 21:1-58.

Shirasu, Y. 1965. Comparative Carcinogenicity of 4-Nitroquinoline 1-Oxide and 4-Hydroxyaminoquinoline 1-Oxide in Three Strains of Mice. Proc. Soc. Exptl. Biol. Med. 118:812-814.

Shubik, P. 1950. The Growth Potentialities of Induced Skin Tumors in Mice. The Effects of Different Methods of Chemical Carcinogenesis. Cancer Res. 10:713-717.

Shubik, P. 1972. The Use of the Syrian Golden Hamster in Chronic Toxicity Testing. Prog. Exp. Tumor Res. 16:176-184.

Silverstone, H., and A. Tannenbaum. 1951. Proportion of Dietary Protein and the Formation of Spontaneous Hepatomas in the Mouse. Cancer Res. 11:442-446.

Smyth, H., C. Carpenter, C. Weil, and J. King. 1970. Experimental Toxicity of Sodium 2-Ethylhexyl Sulfate. Toxicol. Appl. Pharmacol. 17:53-59.

Snell, K. C. 1965. Spontaneous Lesions of the Rat. In: The Pathology of Laboratory Animals, W. E. Ribelin and J. R. McCoy, eds. Charles C. Thomas, Publisher, Springfield, Illinois.

Solt, D. B., A. Medline, and E. Farber. 1977. Rapid Emergence of Carcinogen-Induced Hyperplastic Lesions in a New Model for the Sequential Analysis of Liver Carcinogenesis. Am. J. Path. 88:595-618.

Sontag, J. M., N. P. Page, and U. Saffiotti. 1976. Guidelines for Carcinogen Bioassay in Small Rodents. NCI-CG-TR-1, February. Bethesda, Maryland.

Spatz, M., and G. Laqueur. 1967. Transplacental Induction of Tumors in Sprague-Dawley Rats with Crude Cyad Material. J. Natl. Cancer Inst. 38:233-245.

Spicer, S., L. Chakrin, and J. Wardell, Jr. 1974. Effect of Chronic Sulfur Dioxide Inhalation on the Carbohydrate Histochemistry and Histology of the Canine Respiratory Tract. Am. Rev. Respir. Dis. 110:13-24.

Spitz, S., W. H. Maguigan, and K. Dobriner. 1950. The Carcinogenic Action of Benzidine. Cancer 3:789-804.

Steinmuller, D., L. A. Dillingham, and R. T. Prehn. 1969. Lack of Carcinogenic Activity of 3-Methylcholanthrene in the Squirrel Monkey. J. Natl. Cancer Inst. 43:1175-1180.

Stevens, L. C. 1973. A New Inbred Subline of Mice (129/terSv) with a High Incidence of Spontaneous Congenital Testicular Teratomas. J. Natl. Cancer Inst. 50:235-242.

Stevenson, D. 1979. Current Problems in the Choice of Animals for Toxicity Testing. J. Toxicol. Environ. Health 5:9-15.

Stoltz, D. R., L. A. Poirer, C. C. Irving, H. F. Stich, J. H. Weisburger, and H. C. Frice. 1974. Evaluation of Short-Term Tests for Carcinogenicity. Tox. Appl. Pharmacol. 29:157-180.

Street, A. 1970. Biochemical Tests in Toxicology. In: Methods in Toxicology, G. Paget, ed. F. A. Davis Company, Philadelphia. pp. 313-337.

Strong, L. C., and H. Matsunaga. 1975. An Inverse Correlation (Dose-Response) Between 5-Methyl-Cyiidine and the Fate of Spontaneous Tumors in Mice. Cytobios 12:13-18.

Sugiura, K., W. E. Smith, and D. A. Sunderland. 1956. Experimental Production of Carcinoma in Rhesus Monkeys. Cancer Res. 16:951.

Sumi, N., D. Stavrou, H. Frohberg, and G. Jochmann. 1976. The Incidence of Spontaneous Tumors of the Central Nervous System of Wistar Rats. Arch. Toxicol. 35:1-13.

Sunderman, F. W., Jr. 1971. Metal Carcinogenesis in Experimental Animals. Food Cosmet. Toxicol. 9:105-120.

Svenberg, J., A. Kostner, W. Wechsler, and R. Denlinger. 1972. Quantitative Aspects of Transplacental Tumor Induction with Ethylnitrosourea in Rats. Cancer Res. 32:2656-2660.

Swenberg, J. A., G. L. Petzold, and P. R. Harbach. 1976. In Vitro DNA Damage/Alkaline Elution Assay for Predicting Carcinogenic Potential. Biochem. Biophys. Res. Comm. 72:732-738.

Syndor, K. L. 1973. A Preliminary Investigation of the Susceptibility to Sarcoma Induction by Low Doses of B(*a*)P in Nine Strains of Inbred Male Rats. Proceedings Tobacco and Health Workshop Conf., 4th University of Kentucky Tobacco and Health Research Institute.

Syndor, K. L., O. Butenandt, F. P. Brillantes, and C. Huggins. 1962. Race - Strain Factor Related to Hydrocarbon-Induced Mammary Cancer in Rats. J. Natl. Cancer Inst. 29:805-814.

Tannenbaum, A., and H. Silverstone. 1949. The Influence of the Degree of Caloric Restriction on the Formation of Skin Tumors and Hepatomas in Mice. Cancer Res. 9:724-727.

Tatematsu, M., T. Shirai, H. Tsuda, Y. Miyata, Y. Shinohara, and N. Ito. 1977. Rapid Production of Hyperplastic Liver Nodules in Rats Treated with Carcinogenic Chemicals: A New Approach for an *In Vivo* Short-Term Screening Test for Hepatocarcinogens. Gann 68:499-507.

Terracini, B., P. N. Magee, and J. M. Barnes. 1967. Hepatic Pathology in Rats on Low Dietary Levels of Dimethylnitrosamine. Br. J. Cancer 21:559-565.

Terracini, B., G. Palestro, M. Gigliardi, and R. Montesano. 1966. Carcinogenicity of Dimethylnitrosamine in Swiss Mice. Br. J. Cancer 20: 871-876.

Terracini, B., M. C. Testa, J. R. Cabral, and N. Day. 1973. The Effects of Long-Term Feeding of DDT to BALB/c Mice. Int. J. Cancer 11:747-764.

Thiery, M., and M. van Gijsegem. 1965. Species and Strain Differences in the Susceptibility of the Cervico-Vaginal Squamous Epithel. to 3,4-Benzo(*a*)pyrene. Br. J. Cancer 19:418-429.

Tomatis, L., C. Agthe, H. Bartsch, J. Huff, R. Montesano, R. Saracci, E. Walker, and J. Wilbourn. 1978. Evaluation of the Carcinogenicty of Chemicals: A Review of the Monograph Program of the International Agency for Research on Cancer (1971 to 1977). Cancer Res. 38:877-885.

Tomatis, L., G. Della Porta, and P. Shubik. 1961. Urinary Bladder and Liver Cell Tumors Induced in Hamsters with *o*-Aminoazotoluene. Cancer Res. 21:1513-1517.

Tomatis, L., and V. Turusov. 1975. Studies on the Carcinogenicity of DDT. Gann Monograph on Cancer Res. 17:219-241.

Tomatis, L., V. Turusov, D. Guibbert, B. Duperray, C. Malaveille, and H. Pacheco. 1971. Transplacental Carcinogenic Effect of 3-Methylcholanthrene in Mice and Its Quantitation in Fetal Tissues. J. Natl. Cancer Inst. 47:645-651.

Torkelson, T., B. Leong, R. Kociba, W. Richter, and P. Gehring. 1974. Lack of Manifestation of Toxicity in Rats Inhaling 111 ppm 1,4-Dioxane for Two Years. Toxicol. Appl. Pharmacol. 29:86.

Toth, B. 1968. A Critical Review of Experiments in Chemical Carcinogenesis Using Newborn Animals. Cancer Res. 28:727-738.

Toth, B., P. Magee, and P. Shubik. 1964. Carcinogenesis Study with Dimethylnitrosamine Administered Orally to Adult and Subcutaneously to Newborn BALB/c Mice. Cancer Res. 24:1712-1721.

Toth, B., H. Rappaport, and P. Shubik. 1963. Influence of Dose and Age on the Induction of Malignant Lymphomas and Other Tumors by 7,12-Dimethylbenz(*a*)anthracene in Swiss Mice. J. Natl. Cancer Inst. 30:723-741.

Toth, B., and P. Shubik. 1963. Carcinogenesis in Lewis Rats Injected at Birth with 7,12-Dimethylbenz(*a*)anthracene. Br. J. Cancer 17:540-545.

Turner, F. C. 1941. Sarcomas at the Site of Subcutaneously Implanted Bakelite Discs in Rats. J. Natl. Cancer Inst. 2:81.

Turusov, V., L. Tomatis, D. Guibbert, B. Duperray, and H. Pacheco. 1973. The Effect of Prenatal Exposure of Mice to Methylcholanthrene Combined with the Neonatal Administration of Diethylnitrosamine. In: Transplacental Carcinogenesis, L. Tomatis and U. Mohr, eds. Scientific Publication No. 4, International Agency for Research on Cancer, Lyon, France. pp. 84-91.

U.S. Food and Drug Administration. 1971. Advisory Committee on Protocols for Safety Evaluation, Panel on Carcinogenesis Report on Cancer Testing in the Safety Evaluation of Food Additives and Pesticides. Toxicol. Appl. Pharmacol. 20:419-438.

Verschuuren, H., R. Kroes, E. Dentonkelaar, J. Berkvens, P. Hellman, and G. Van Esch. 1975. Long-Term Toxicity and Reproduction Studies with Metaldehyde in Rats. Toxicology 4:97-115.

Verschuuren, H., R., Kroes, and G. Van Esch. 1973. Toxicity Studies on Tetrasul. I. Acute, Long-Term, and Reproduction Studies. Toxicology 1:63-78.

Vesselinovitch, S. 1973. Comparative Studies on Perinatal Carcinogenesis. In: Transplacental Carcinogenesis, L. Tomatis and U. Mohr, eds. Scientific Publication No. 4, International Agency for Research on Cancer, Lyon, France. pp. 14-22.

Vesselinovitch, S. D., A. P. Kyriazis, N. Mihailovich, and K.V.N. Rao. 1975. Conditions Modifying Development of Tumors in Mice at Various Sites by Benzo(*a*)pyrene. Cancer Res. 35:3948-2953.

Vesselinovitch, S., and N. Mihailovich. 1966. Significance of Newborn Age and Dose of Urethan in Leukemogenesis. Cancer Res. 26:1633-1637.

Vesselinovitch, S, N. Mihailovitch, and L. Itze. 1970. Comparative Studies on the Kinetics of the Neoplastic Competence in Mice. Cancer Res. 30:2548-2551.

Vesselinovitch, S., N. Mihailovich, G. Wogan, L. Lombard, and K. Rao. 1972. Aflatoxin B_1, a Hepatocarcinogen in the Infant Mouse. Cancer Res. 32:2289-2291.

Vesselinovitch, S., K. Rao, and N. Mihailovich. 1975. Factors Modulating Benzidine Carcinogenicity Bioassay. Cancer Res. 35:2814-2819.

Vlahakis, G., W. E. Heston, and G. H. Smith. 1970. Strain $C3H\text{-}A^{vy}fB$ Mice: Ninety Percent Incidence of Mammary Tumors Transmitted by Either Parent. Science 170:185-187.

Walpole, A. L., M.H.C. Williams, and D. C. Roberts. 1952. The Carcinogenic Action of 4-Aminodiphenyl and 3:2'Dimethyl-4-Aminodiphenyl. Br. J. Ind. Med. 9:255-263.

Walpole, A. L., M.H.C. Williams, and D. C. Roberts. 1954. Tumors of the Urinary Bladder in Dogs After Ingestion of 4-Aminodiphenyl. Br. J. Ind. Med. 11:105-109.

Watabe, H. 1971. Early Appearance of Embryonic α-Globulin in Rat Serum During Carcinogenesis with 4-Dimethylaminobenzene. Cancer Res. 31: 1192-1194.

Watanabe, P., J. Young, and P. Gehring. 1977. The Importance of Non-Linear (Dose-Dependent) Pharmacokinetics in Hazard Assessment. J. Environ. Pathol. Toxicol. 1:147-159.

Weil, C. 1962. Applications of Methods of Statistical Analysis to Efficient Repeated-Dose Toxicological Tests: 1. General Considerations and Problems Involved. Sex Differences in Rat Liver and Kidney Weights. Toxicol. Appl. Pharmacol. 4:561-571.

Weil, C. 1972. Guidelines for Experiments to Predict the Degree of Safety of a Material for Man. Toxicol. Appl. Pharmacol. 21:194-199.

Weil, C. 1973. Experimental Design and Interpretation of Data from Prolonged Toxicity Studies. Pharmacology and the Future of Man. Proc. 5th Int. Congr. Pharmacol., San Francisco, 1972, Vol. 2: Toxicological Problems, Karger, New York. pp. 4-12.

Weil, C., and C. Carpenter. 1969. Abnormal Values in Control Groups During Repeated-Dose Toxicologic Studies. Toxicol. Appl. Pharmacol. 14:335-339.

Weil, C., and D. McCollister. 1963. Safety Evaluation of Chemicals. Relationships Between Short- and Long-Term Feeding Studies in Designing and Effective Toxicity Test. Agric. Food Chem. 11:486-491.

Weil, C., M. Woodside, H. Smyth, Jr., and C. Carpenter. 1971. Results of Feeding Propylene Glycol in the Diet of Dogs for Two Years. Food Cosmet. Toxicol. 9:479-490.

Weisburger, E. K. 1977. Carcinogenicity Studies on Halogenated Hydrocarbons. Environ. Health Perspect. 21:7-16.

Weisburger, J. H. 1976. Bioassays and Tests for Chemical Carcinogens. In: Chemical Carcinogens, American Chemical Society, Monograph 173, C. E. Searle, ed. Washington, D.C. pp. 1-23.

Weisburger, J., M. Klein, E. Weisburger, R. Glass, G. Woodard, and M. Gronin. 1970. Comparison of the Effect of the Carcinogen *N*-Hydroxyl-*N*-2-Fluorenylacetamide in Infant and Weanling Rats. J. Natl. Cancer Inst. 45:29-35.

Weisburger, J. H., and E. K. Weisburger. 1967. Tests for Chemical Carcinogens. From: Methods in Cancer Res., Vol. I., H. Busch, ed. Academic Press, New York. pp. 307-398.

Welsch, C. W. 1976. Interaction of Estrogen and Prolactin in Spontaneous Mammary Tumorigenesis of the Mouse. J. Toxicol. Environ. Health Suppl. 1:161-175.

Wigley, C. B., J. Amos, and P. Brookes. 1978. Different Tumours Induced by Benzo(*a*)pyrene and Its 7,8-Dihydrodiol Injected into Adult Mouse Salivary Gland. Br. J. Cancer 37:657-661.

Winstead, J. 1978. What Kinds of Tests Are Prerequisite to Labeling a Compound as Toxic? Int. J. Occup. Health Saf. 47(3):26-28.

Wislocki, P. G., R. L. Chang, A. W. Wood, W. Levin, H. Yagi, O. Hernandez, H. D. Mah, P. M. Dansette, D. M. Jerina, and A. H. Conney. 1977. High Carcinogenicity of 2-Hydroxybenzo(*a*)pyrene on Mouse Skin. Cancer Res. 37:2608-2611.

Wogan, G. N., and R. C. Shank. 1971. Toxicity and Carcinogenicity of Aflatoxins. In: Advances in Environmental Science and Technology, J. N. Pitts and R. L. Metcalf, eds., John Wiley & Sons, Inc., New York. pp. 321-350.

Wold, J., J. Welles, N. Owen, W. Gibson, and D. Morton. 1978. Toxicologic Evaluation of Cefamandole Nafate in Laboratory Animals. J. Infect. Dis. 137:551-559.

Womack, J. 1979. Genetic Constitution and Response to Toxic Chemicals — An Overview. J. Toxicol. Environ. Health 5:49-51.

Woodard, G., M. Woodard, W. McNeely, P. Kovacs, and M. Cronin. 1973. Xanthan Gum: Safety Evaluation by Two-Year Feeding Studies in Rats and Dogs, and a Three-Generation Reproductive Study in Rats. Toxicol. Appl. Pharmacol. 24:30-36.

Worden, A., K. Rivett, D. Edwards, A. Street, and A. Newman. 1975. Long-Term Feeding Study on Disodium 5'-Ribonucleotide in Dogs. Toxicology 3:344-347.

World Health Organization. 1978. Principles and Methods for Evaluating the Toxicity of Chemicals. Part 1. Geneva. 274 pp.

Wurzner, H., E. Lindstrom, L. Vualaz, and H. Luginbuhl. 1977. A 2-Year Feeding Study of Instant Coffees in Rats. I. Body Weight, Food Consumption, Haematological Parameters and Plasma Chemistry. Food Cosmet. Toxicol. 15:7-16.

Yoshida, T. 1932. Uber die Experimantelle Erzeugung von Hepatom durch die Futterung mit o-Amido-azotoluol. Proc. Imp. Acad. Japan 8:464-467.

Zabezhinskii, M. A. 1970. Effectiveness of Inhalation as a Method of Administration of Atomized Carcinogens. Bull. Exptl. Biol. and Med. 69:68-70.

Zackheim, H. S. 1964. Comparative Cutaneous Carcinogenesis in the Rat. Oncologia 17:236-246.

Zbinden, G. 1973. Formal Toxicology. In: Progress in Toxicology, Special Topics, Vol. 1. Springer-Verlag, New York. pp. 4-27.

Zoller, M., S. Matzku, and K. Goerttler. 1978. High Incidence of Spontaneous Transplantable Tumours in BDX Rats. Brit. J. Cancer 37:61-66.

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16. ABSTRACT

This document is the first of a two-part literature analysis of parameters associated with the various toxicity testing methods (test animal selection, pathology requirements, etc.) Acute, subchronic, chronic, and carcinogenic testing methods are covered; a discussion of some basic experimental considerations is also included. This report was prepared for the purpose of assisting and supporting the U.S. Environmental Protection Agency in its efforts to develop guidelines for more efficient and economical testing procedures.

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